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DEDICATION

We dedicate this book to our son Niklas Sebastian, the absolute joy and love of our life.

PREFACE

Our reasons for deciding to publish an entire book devoted to the role of Notch signalling in embryology and cancer can be found in the rapid and extensive advances currently being made in this exciting field of research. In recent years, our knowledge about the cellular and molecular basis of Notch signalling has dramatically increased, underlining the importance of this signalling pathway for a broad variety of physiologic and pathophysiologic conditions, including embryology and cancer.

The goal of this volume is to comprehensively cover a highly readable overview on our present knowledge of the role of Notch signalling for embryology and cancer, with a focus on new findings in molecular biology. Topics that are discussed in-depth by leading researchers and clinicians range from the newest findings in cellular and molecular pathology to new concepts for prophylaxis and treatment of cancer. Experts in the field as well as scientists and health care professionals not intimately involved in these specialized areas are provided with the most significant and timely information related to these topics. It is the aim of this book to summarize essential up-to-date information for every clinician or scientist interested in the role of Notch signalling in embryology and cancer. We hope that this volume will be adopted as the standard text in this area of science and will stimulate new interest in this topic. We further hope that this compendium will serve as both a source for current researchers and a guide to stimulate and assist those in related disciplines to enter this exciting field of research.

Our goal in planning this book was to bring the diverse scientific and clinical fields together in one definitive and up-to-date volume. We are therefore especially pleased with the roster of authors who have contributed to this book. All the chapters are written by authors who are leading experts in their respective research areas, and we are very grateful for their willingness to contribute to this book. We would also like to express my thanks to Ron Landes, Cynthia Conomos, Celeste Carlton, Erin O`Brien, and all the other members of the Landes Bioscience staff for their expertise, diligence and patience in helping us complete this work.

Sandra and Jörg Reichrath

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CHAPTER 1

THE MOLECULAR BASIS OF NOTCH SIGNALING: A Brief Overview

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Abstract: The Notch signaling pathway is evolutionarily conserved and has been associated with numerous developmental processes, including stem cell maintenance and adult tissue homeostasis. Notably, both abnormal increases and deficiencies of Notch signaling result in human developmental anomalies and cancer development implying that the precise regulation of the intensity and duration of Notch signals is imperative. Numerous studies have demonstrated that the aberrant gain or loss of Notch signaling pathway components is critically linked to multiple human diseases. In this chapter, we will briefly summarize the molecular basis of Notch signaling, focusing on the modulation of Notch signals, and its developmental outcomes including vessel formation and the onset of cancer.

INTRODUCTION

The Notch signaling pathway is evolutionarily conserved and has been associated with numerous developmental processes, including stem cell maintenance and adult tissue homeostasis.[1,2] Notably, both abnormal increases and deficiencies of Notch signaling result in human developmental anomalies and cancer development, implying that the precise regulation of the intensity and duration of Notch signals is imperative.[3] Notch receptor-mediated signals have a central role in cell fate decisions and regulate the differentiation of epithelial, neuronal, blood, bone, muscle and endothelial cells.[4,5]

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Additionally, Notch signaling is involved in other biological processes such as arterial cell-fate determination during embryonic development, or the control of pathological angiogenesis by regulating the selection of endothelial tip and stalk cells in sprouting blood vessels during adult homeostasis.[6-9] Numerous studies have demonstrated that the aberrant gain or loss of Notch signaling pathway components is critically linked to multiple human diseases. For example, the loss of function of Notch signaling components has been involved in inherited genetic disorders such as Alagille syndrome, Spondylocostal dysostosis (SCD) and Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy,[10,11] as well as in the onset of cancer. Up-regulation of Notch activity on the other side has been reported to be involved in the development of leukemias such as T-cell acute lymphoblastic leukemia (T-ALL).[12-14] In this chapter, we will briefly summarize the molecular basis of Notch signaling, focusing on the modulation of Notch signals, and its developmental outcomes including vessel formation and the onset of cancer.

BASIC MECHANISMS OF NOTCH SIGNALING PATHWAYS

Notch signaling is initiated by the interaction between a Notch ligand and a Notch receptor expressed on the surface of a neighboring cell. In mammals, the Notch pathway consists of four Notch transmembrane receptors (Notch-1 to Notch-4), which display both redundant and unique functions, and five Type I transmembrane ligands, including three Delta-like proteins (Dll-1, 2 and 4) and two Jagged proteins (Jagged-1 and Jagged-2). Notch receptors are large single-pass Type I transmembrane proteins that receive signals from transmembrane ligands which are located in neighboring cells.[15] (Figs. 1A and 1B).

As shown in Figure 2, canonical Notch signaling begins with the binding of the ligands DSL (Delta, Serrate, Lag2) to the Notch receptors at the cell surface. Upon Notch ligand binding, a two-step proteolysis cleavage process within the juxtamembrane region and transmembrane domain of the Notch receptor mediated by ADAM/TACE metalloproteases[16-18] is initiated, resulting in the release of the Notch intracellular domain (NICD) into the cytoplasm.[5,19,20] The NICD enters the nucleus, where it associates with a DNA binding protein, to assemble a transcriptional complex consisting of CSL (CBF1/RBPJK in mammals, Su(H) in flies, and LAG-1 in worms), the ICN, and a co-activator protein of the Mastermind (MAM)/Lag-3 family.[3,21-23] These then activate downstream target genes via the recruitment of additional co-activators such as p300 and mediate transcriptional activation from chromatin templates by the ICN (Fig. 2).[24,25] The best-characterized Notch targets are members of the "Hairy and enhancer-of-split" (HES), and "Hairy and enhancer-of-split-related" (HEY, HESR, HRT, or CHF) gene families.[26] These proteins dominantly act as transcriptional repressors, either by direct binding to specific regions such as E- and N-boxes and the recruitment of corepressors including Groucho or by distinct mechanisms which are independent of direct DNA binding.[27,28] Many of these genes repress genes that are required for cell differentiation. One of the most studied functions of Notch signaling is lateral inhibition by blocking surrounding cells from adopting the same fate.

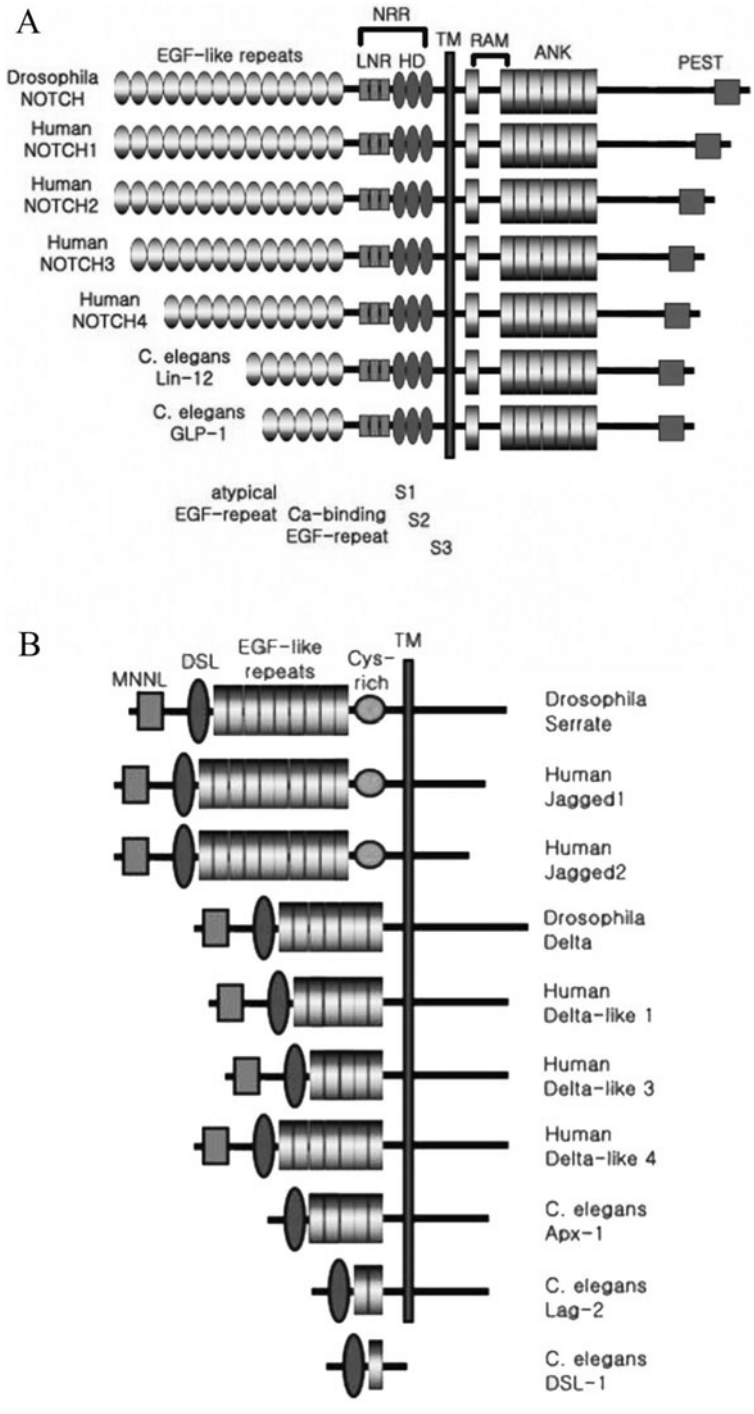


Figure 1. A,B) Domain organization of Notch receptors and DSL-family ligands.

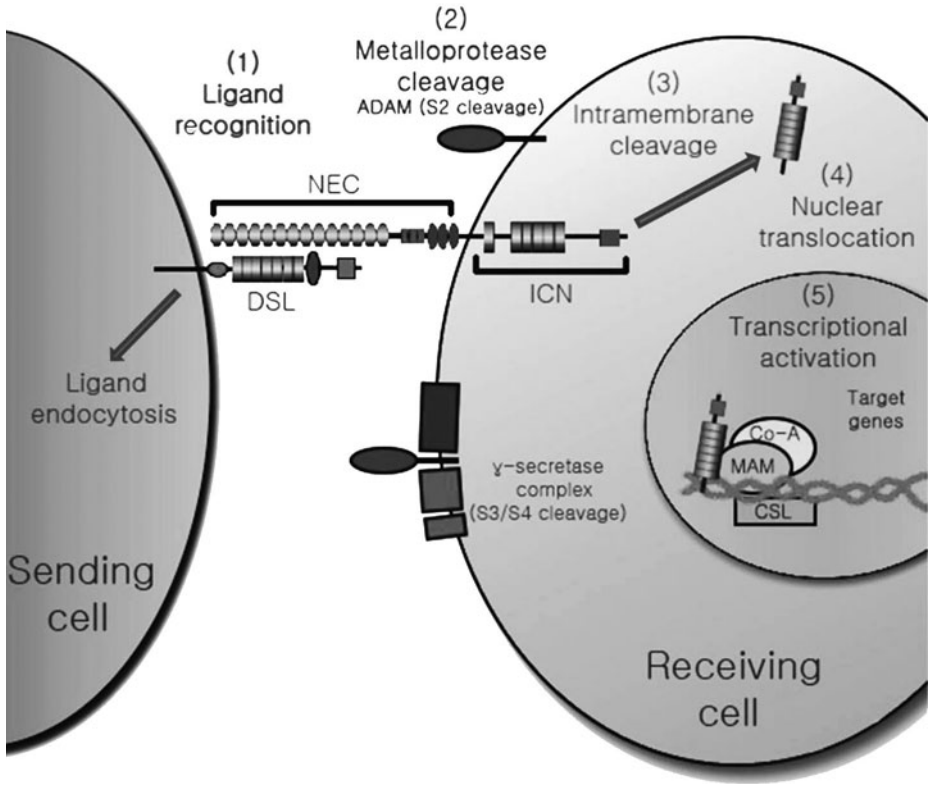


Figure 2. Notch signaling pathway; Model for the major signal cascades. 1) Signal initiation by the binding of Notch ligand. 2) Metalloprotease cleavage (MP) at site. 3) Cleavage of Notch. 4) Nuclear translocation. 5) Transcriptional activation.

MODULATION OF NOTCH SIGNAL TRANSDUCTION

One basic method of regulation of Notch signaling is to restrict Notch ligand or Notch receptor availability, either spatially or temporally; however differential expression information of the ligands and receptors is not enough to explain the distinct Notch signaling effects in different cellular contexts. There are numerous proteins that modulate Notch signaling. Endocytic trafficking of the DSL ligands plays a pivotal role in enhancing their signaling activity.[29,30] One way of modulation of Notch signaling consists in the control of the cleavage steps by E3 ligases Neuralized and Mindbomb,[31,32] facilitating endocytosis of Notch ligands. Other modulators of Notch signaling act to modify ligand responsiveness via posttranslational modification of Notch by the glycosyltranferases Fringe or Rumi,[33-37] as well by modulating ligands and NICD turnover via Sel-10 and related F-box proteins.[38,39] NICD affects transcription with the help of its partner CSL, directing NICD to specific target genes through interaction with a conserved WxP motif in its RAM domain. The NICD/CSL complex also seems to affect nuclear events by competing with the transcription co-activator Mastermind (MAM; MAML in mammals). Based on studies in flies, loss of the CSL protein Su(H) results in the activation of Notch

target genes, further showing that Su(H) is associated with transcription repression via recruitment of the transcription corepressors, Hair-less/CtBP, SKIP and Gro/TLE. The CSL protein Su(H) can silence transcription by recruiting Asf1, a histone chaperone, mediating the transcriptional switch from activation to repression.[40] Recent studies have shown that NICD can interact with various transcriptional cofactors, SMADs, NFκB (Nuclear factor kappa-B) and HIF1α (Hypoxia-inducible factor 1-alpha), reflecting binding of adjacent enhancer-associated complexes. Other direct targets include CyclinD1, p21 (Cyclin-dependent kinase inhibitor 1), GFAP (Glial fibrillary acidic protein), Nodal, Myc, PTEN (Phosphatase and tensin homolog), ephrinB2 and SMA (Smooth muscle α-actin).

Notch activity does not rely on secondary messengers for signal amplification, but indeed seems to be modulated by glycosylation, differential endocytic trafficking of ligand and receptor, and ubiquitin-dependent degradation, sorting and recycling. The complex sugar modifications of the Notch receptors, mainly via *O*-fucose and *O*-glucoseglycans facilitate proper Notch folding in the ER, but the exact biological role of sugars in regards to the activation of Notch signaling is still not well defined. After translation, the extracellular domain of Notch is predominantly fucosylated. *O*-fucosyltransferases can modify the EGF (Epidermal growth factor) consensus repeats. The fucosylation does appear to be critical for Fringe glycosyltrasferase-regulated Notch signaling, but the precise role of *O*-fucosylation in Notch activation awaits further clarification. The *O*-fucose residues of the Notch extracellular domain can be further modified with N-glycans via Fringe, a β1,3-N-acetylglucosaminyltransferase.[41,42] In flies, the sugar modification of Fringe facilitates Delta ligand binding to Notch and negatively affects its binding ability to Serrate.[33] Both the conserved N-terminal and the DSL domains of ligands appear to participate in optimal binding to Notch.[43,44]

NOTCH SIGNALING IN VASCULAR DEVELOPMENT

In human vascular diseases, haploinsufficiency of the Jag-1gene is closely related to the Alagilles syndrome, characterized by various pleiotropic developmental problems with accompanying features of congenital heart defects and cardiovascular anomalies.[45] A mutation in the Notch-3 gene is directly linked to a severe human degenerative vascular disease termed Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL). Similarly, mice with targeted deletion of Notch family genes including Notch receptors or their ligands exhibit diverse defects during blood vessel formation or arterio-venous specification[46] and show impaired proliferation and migration of endothelial cells (ECs).[47] Mice lacking Notch-1, Notch-3,[48] Notch-4,[49,50] Dll-4[51] or Jag-1[52] show severe vascular defects in angiogenesis and vascular development. The targeted deletion of Notch transcriptional targets Hey1 and Hey2 leads to impaired vascular development in mice, with vessels still forming de novo but remaining small and failing to mature.[53] In the zebrafish, knockdown of Notch3, RBP-J, Mindbomb and Gridlock can cause a deregulation of arterial and venous specification of endothelial cells.[54,55] To balance the number and proper assembly of arterial and venous endothelial cells into distinct vascular structures, Notch signaling regulates downstream targets, including VEGFR2 (Vascular endothelial growth factor receptor 2), MAPK (Mitogen-activated protein kinase), ephrinB2, and EphB4.[47,56,57] EphrinB2, which shows an expression restricted to arterial endothelial cells, seems to be a direct target of Notch, suggesting that active Notch signaling controls arterial specification of endothelial cells.

In contrast, COUP-TFII, a venous transcription factor, has been reported to actively repress the arterial marker Neuropilin-1 and inhibit the Notch signaling cascade. Accordingly, targeted deletion of COUP-TFII, leads to the expansion of the arterial cell fate during early embryonic development.[46]

The dynamic interplay between VEGF and Notch signaling plays a central role both in the initial formation and patterning of the mesodermal components of the developing vasculature as well as in subsequent specification and maturation processes such as venous to arterial differentiation. A precise understanding of the dynamics of VEGF/Notch signaling under physiological and pathological settings will thus greatly enhance our understanding of the mechanisms controlling vascular pattering. VEGFs and their receptors are major regulators of angiogenesis and vascular permeability, modulating the proliferation, branching, migration, and survival of endothelial cells during normal development and tumor angiogenesis.

Endothelial cells respond to VEGFR2 activation with the formation of dynamic protrusions of actin rich filopodia, through repression of Dll4 or Notch, suggesting that VEGF and Notch feedback signals are sufficient to create the dynamic sprouting of tip and stalk cells in response to VEGF stimulation. Most tip cells express Dll4, up-regulated in response to hypoxia. VEGF is able to stimulate Dll4 expression via the phosphoinositide 3-kinase (PI3K)/Akt pathway downstream of VEGFR2.[58] VEGFR2 dependent Dll4/Notch signaling is in part mediated by the basic helix-loop-helix transcription factor HESR1[59] and the forkhead transcription factors FOXC1 and FOXC2.[60,61] A schematic illustration of a negative feedback loop to control tip-stalk cell specification is proposed (Fig. 3). VEGF-A promotes Dll4 expression, and Notch signaling activated by DLL4 leads to the downregulation of VEGF receptors, illustrating that adequate concentration of VEGF-A can alter tip and stalk cells.[62] Notch signaling was actually reported to regulate transcriptional steps of Flt1 (Vascular endothelial growth factor receptor 1),[63] Kdr (Vascular endothelial growth factor receptor 2),[64,65] Flt4 (Vascular endothelial growth factor receptor 3)[66] and Nrp1 (Neuropilin 1)[67] expression.

The precise control of endothelial cell proliferation and junctional re-arrangement is crucial for the proper stabilization of a forming functional vascular network. Recently, a downstream molecule of Notch signaling, Nrarp (Notch-regulated ankyrin repeat protein), was reported to stabilize nascent blood vessels during retinal angiogenesis through a cross talk between Notch/Nrarp and Wnt signaling.[68-70] The β-catenin/Lef1 complex, a pivotal component of Wnt signaling, activates endothelial cell proliferation via the direct activation of Cyclin D1, with Nrarp playing a critical role in the modulation of the output of Notch and Wnt signals.

NOTCH SIGNALING IN CANCER DEVELOPMENT

Several studies using mouse tumor model systems suggest an involvement of Dll4/Notch signaling in tumor angiogenesis, proposing a role of Notch signaling in the co-ordination and balance of the tip and stalk cell number, required for effective vascular patterning and the control of the branching frequency of tumor blood vessels.[71,72] Dll4 is strongly expressed in renal cell[73] and in invasive bladder carcinomas.[74] Recently, several groups have investigated whether inhibition of the Dll4/Notch pathway might affect tumor angiogenesis and growth. The systemic or local administration of Dll4-neutralizing antibodies or modified Dll4 clearly shows that the inhibition of Dll4-mediated Notch

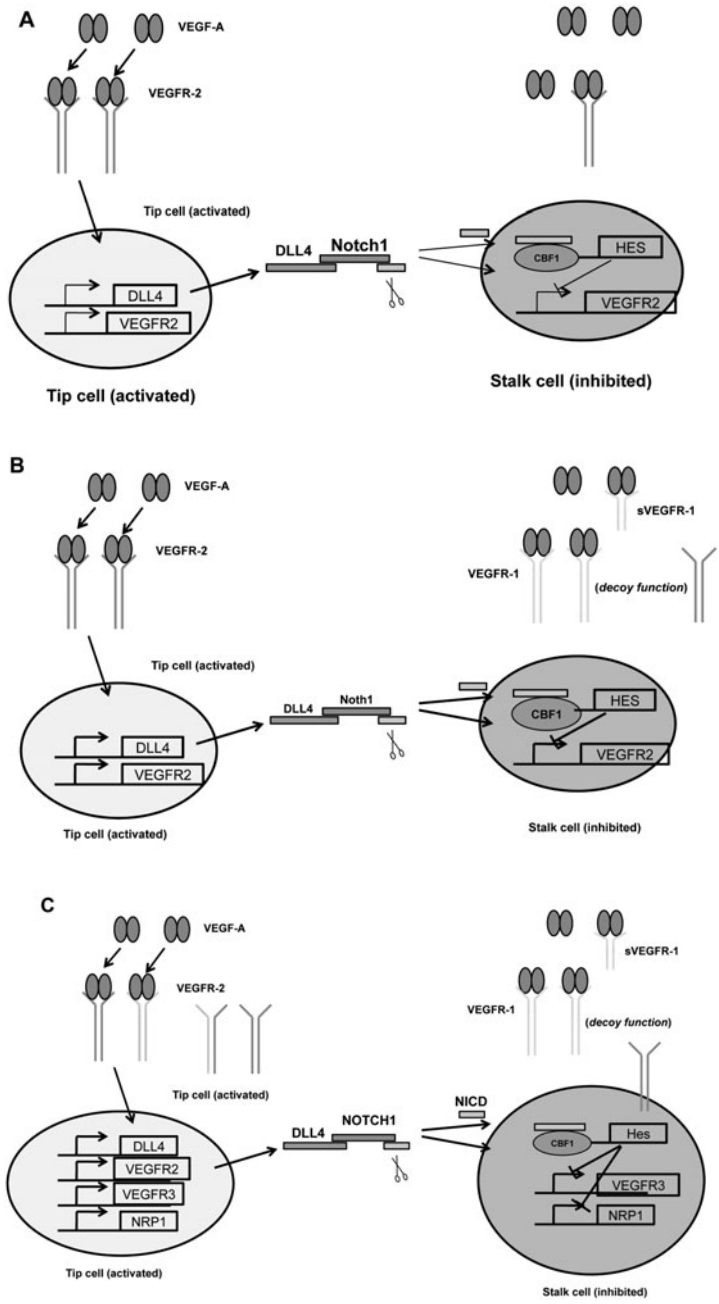


Figure 3. Schematic illustration of possible mechanisms of tip stalk cell modulation by different components of the VEGF and Notch signaling pathways. A) VEGF-A binds and activates VEGFR2 in the tip cell, leading to the activation of the Dll4 promoter and the VEGFR2 promoter reinforcing high levels of VEGFR2 receptor expression. B) Additional pathway components are regulated by Notch signaling. C) VEGFR3 and Neuropilin-1 (NRP1) expression is induced in tip cells, prompting net activity of VEGF signaling.

signaling leads to robust tumor-vessel sprouting, indicating that Dll4-Notch signaling is critical for tip- and stalk-cell selection during tumor angiogenesis.

Furthermore, some recent studies indicate that the Dll4/Notch pathway might have a broad and diverse interaction with angiogenic pathways other than VEGF/VEGFR signaling, suggesting that Dll4/Notch inhibition might be beneficial in the treatment of tumors which are either intrinsically less dependent on VEGF or which have progressed and shifted to other angiogenic pathways. Indeed, preclinical studies have shown that blocking Dll4 was effective in inhibiting the growth of tumors that are resistant to VEGF inhibition. Albeit the existence of studies showing that the inhibition of Dll4 delays tumor growth, recent studies have also shown, that the blockage of the Dll4-Notch pathway in mice can induce tumor angiogenesis. This paradoxical phenomenon may be explained by analyzing the functionality of the microvasculature formed by enhanced tumor angiogenesis, which shows overall poor vascular integrity and inefficiently perfuses the tumor, thereby increasing tumor hypoxia. In other words, blocked Dll4 causes the formation of nonfunctional vasculature, resulting in a delay of tumor growth.[75-77] Therefore, Dll4 has become a potential anti-angiogenic therapeutic target. Moreover, when combined with anti-VEGF treatment, inhibition of Dll4 was shown to be even more efficient in controlling tumor growth.[76] Concordantly, Li et al have illustrated that Dll4 expressed in tumor cells activates the Notch signaling pathway in mouse endothelial cells and improves tumor vascular function (Fig. 4).

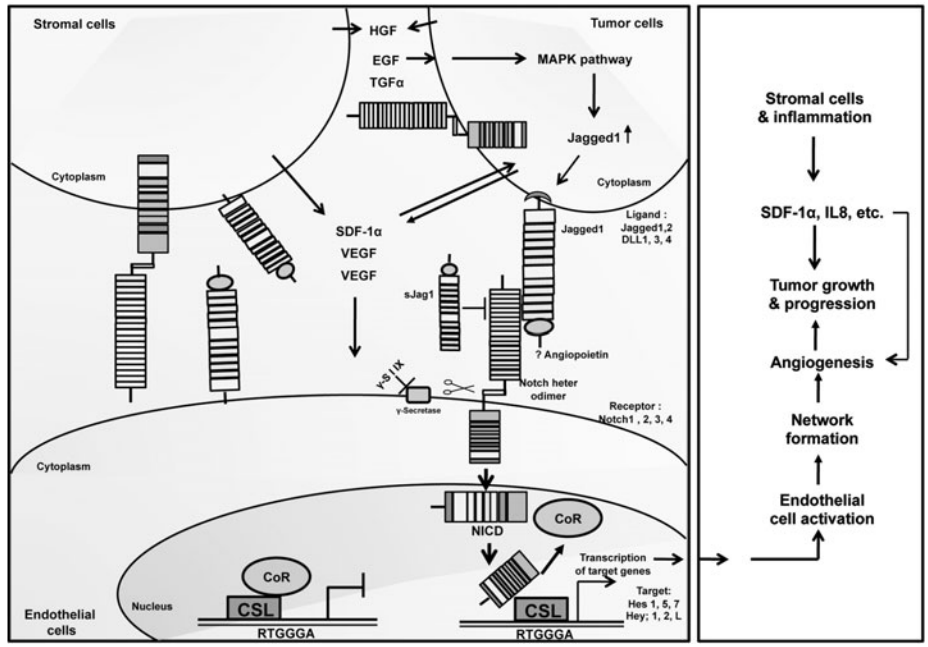


Figure 4. Cross talk between tumor cells, endothelial cells and stromal cells is modulated by the Notch pathway and stimulates tumor angiogenesis.

NOTCH IN SOLID TUMORS

The tumorigenic activity of Notch in breast cancer has been demonstrated using several mouse model systems. In 1987, insertion of the mouse mammary tumor virus (MMTV) into the Notch4 locus, referred to as *int3* in the Czech II mouse strain was revealed.[78] Another study showed that Notch1 was mutated by a MMTV insertion, with the resulting truncated form of Notch1 functioning as an oncogene leading to the development of mammary carcinomas.[79] Parr et al,[80] demonstrated that expression of Notch1 is increased in poorly-differentiated human breast tumors, while an increased level of Notch2 is associated with a higher chance of survival, suggesting a tumor promoting function for Notch1 and a tumor suppressor role of Notch2 in human breast cancers.[80] Many studies have further reported that Notch signaling plays an oncogenic role in breast cancer mainly through its interaction with other signaling pathways involved in mammary tumorigenesis, such as Ras, Erb2, TGF-β (Transforming growth factor beta) and Wnt signaling pathways. For example, 80% of mice overexpressing transgenic human Ras developed mammary tumors, whereas in mice overexpressing simultaneously transgenic Ras and the Notch inhibitor Deltex, only 20% developed mammary tumors, accentuating the co-operative character of Ras and Notch signaling in the development of breast cancer.[81]

Colorectal cancer is the second most common cause of death due to malignancy worldwide.[82] Early stages of colorectal tumors require angiogenesis,[83,84] which is dependent on the increased expression of pro-angiogenic factors (e.g., VEGF-A).[84,85] The Notch ligand, Dll4, is expressed by endothelial cells[86,87] and can be induced by VEGF[88] and hypoxia through HIF-1α.[89] A recent study has found that Dll4 is highly expressed in the endothelium of a large cohort of colon cancers, further showing that its expression is tightly correlated with VEGF and hypoxia.[90]

NOTCH SIGNALING IN EMBRYONIC DEVELOPMENT

For some model organisms it is apparent that Notch signaling is already required during very early stages of embryonic development, such as the formation of the three germ layers. A co-option of Notch signaling during evolution for the use in early cell fate decisions and embryogenesis likely occurred through different mechanisms in the lineages of *C. elegans*, sea urchin, and zebrafish, respectively, but it did not occur in the lineage leading to mammals (Fig. 5). In *C. elegans*, Notch signaling is extensively involved in the earliest cell fate decisions. At the 4-cell stage, the Notch ligand-expressing P2 cell induces Notch signaling in the ABp cell and represses the expression of two Tbox genes, TBX-37 and TBX-38. As a result, ABp descendant cells will adopt a fate that is different from that of ABa descendant cells. At the 12-cell stage, the mesoderm cell stage, the mesoderm cell sends a Notch signal to two of the ABa descendant cells in which Notch signaling co-operates with TBX-37 and TBX-38, making these cells adopt a mesodermal fate.[91]

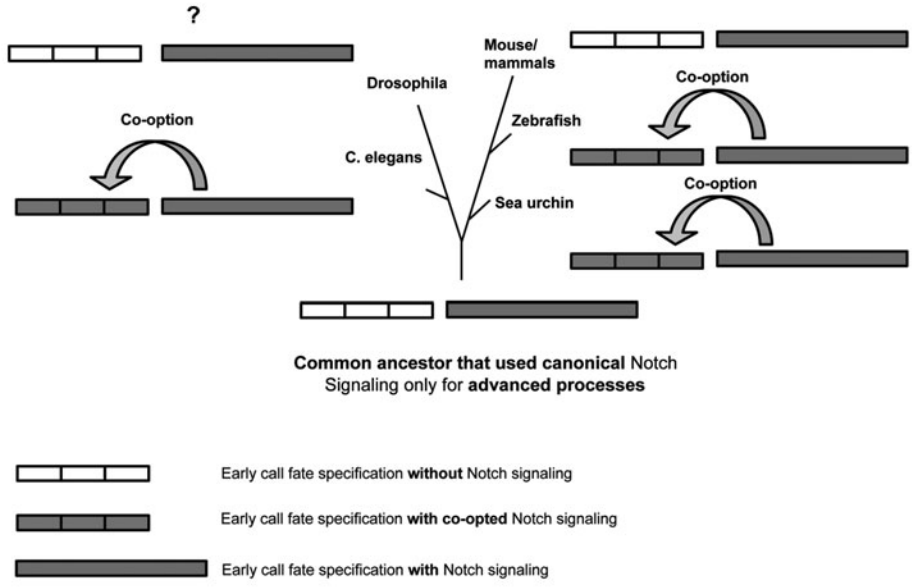


Figure 5. An evolutionary model of Notch signaling in early cell fate specification.

ORIGINS OF ROLES FOR NOTCH SIGNALING IN EARLY EMBRYOGENESIS

The dispensability of canonical Notch signaling for early mouse embryogenesis provides a new insight into the roles of Notch signaling during embryonic development of the metazoans. It is surprising that Notch signaling is not required in mammals, contrasting with the development requirements of several lower organisms. The varied uses of canonical Notch signaling during early development by different organisms raise the possibility that these roles were not inherited from a common ancestor during evolution. In other words, Notch signaling may not have been used for early development in the last common ancestor of the protostomes (including *C. elegans and Drosophila*) and the deuterostomes (including sea urchins, fish and mice). In contrast, Notch signaling might have evolved to function in the common ancestor for segmentation, or other processes that are more advanced than germ layer formation. During subsequent evolution, canonical Notch signaling may have been co-opted differently in different lineages for a new use in early cell differentiation and germ layer formation. The more advanced processes in which Notch was used might have been segmentation or neurogenesis, because the first obvious effect of a loss of global Notch signaling during mouse embryogenesis appears in somites and the neural tube at E8.5.[92-95] A correlation between ontogeny and phylogeny is often observed.

NOTCH SIGNALING IS OFTEN INVOLVED IN MESODERM FORMATION

Although roles for canonical Notch signaling in early embryonic development are diverse, as outlined above, it is notable that Notch signaling seems to facilitate the specification of mesodermal cells during early embryonic development. This might also have arisen through co-option of Notch signaling mechanisms used for more advanced developmental processes. Some key transcription factors, which are commonly used across phyla in mesoderm formation (e.g., GATA family transcription factors, Snail, Twist and Brachyury), can be regulated by Notch signaling. For example, Notch signaling upregulates the expression of Snail and induces epithelial-to-mesenchymal transition,[95,96] a process that is required for mesoderm formation. Notch/Su(H) signaling has been shown to regulate Brachyury expression in Ciona, and to affect the formation of the notochord, a mesodermal structure, during gastrulation.[97] In fact, it was shown earlier that there is a recognition site of Su(H) in the enhancer region of the Ciona Brachyury gene.[98] Notch signaling can also regulate Twist expression and thereby mesodermal segment patterning in *Drosophila.*[99] Finally, activation of Notch signaling in the endoderm of zebrafish has been shown to inhibit the expression of GATA5, a factor that is required for endoderm formation.[100]

CONCLUSION

The precise understanding of Notch signaling is very important; Notch signaling has emerged as a specific and potential therapeutic target in the treatment of cancers. In addition, although the efforts to elucidate the role of Notch signaling with regard to cell/tissue development require detailed knowledge of how the "on" and "off" signals are brought out, these ongoing studies will provide pivotal clues to understanding the molecular signaling cascades governing cell differentiation and tissue development.

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CHAPTER 2

THE ROLE OF ADAMS IN NOTCH SIGNALING

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Abstract: Regulated intramembrane proteolysis (RIP) is a highly conserved signaling paradigm whereby membrane-bound signaling proteins are cleaved in their transmembrane region and then released into the cytoplasm to act as signaling molecules. In most if not all cases intramembrane cleavage is preceded and regulated by a membrane proximal cleavage step called "ectodomain shedding". Here we will review the role of ectodomain shedding in RIP of the NOTCH signaling pathway, a highly conserved cell-cell communication pathway that mediates cell fate decisions during development and in adult tissues.

INTRODUCTION

RIP is a widespread signaling paradigm where transmembrane proteins are cleaved within their transmembrane domain to release a cytosolic signaling fragment that can enter the nucleus to control gene transcription.[1] The RIP is conserved from bacteria to humans[2] and controls many diverse processes from lipid metabolism,[3] the unfolded protein response,[4] Epidermal Growth Factor (EGF) signaling,[5] antigen presentation,[6] production of the beta Amyloid Precursor Protein (APPβ)[7] and cell fate determination by the Notch signaling pathway.[8] There are 4 classes of Intramembrane Cleaving Proteases (I-CLIPs), Aspartyl Proteases, Presenilins (PS) and Signal Peptide Peptidases (SPP), Serine Proteases (Rhomboid) and Metalloproteases (Site-2 Protease (S2P)). No cysteine or threonine proteases have been identified that exhibit I-CLIP activity.[9] I-CLIPs are polytopic membrane proteins with catalytic residues within their transmembrane domains (TMD) that hydrolyze peptide bonds within the lipid bilayer.[10] PS and Rhomboids

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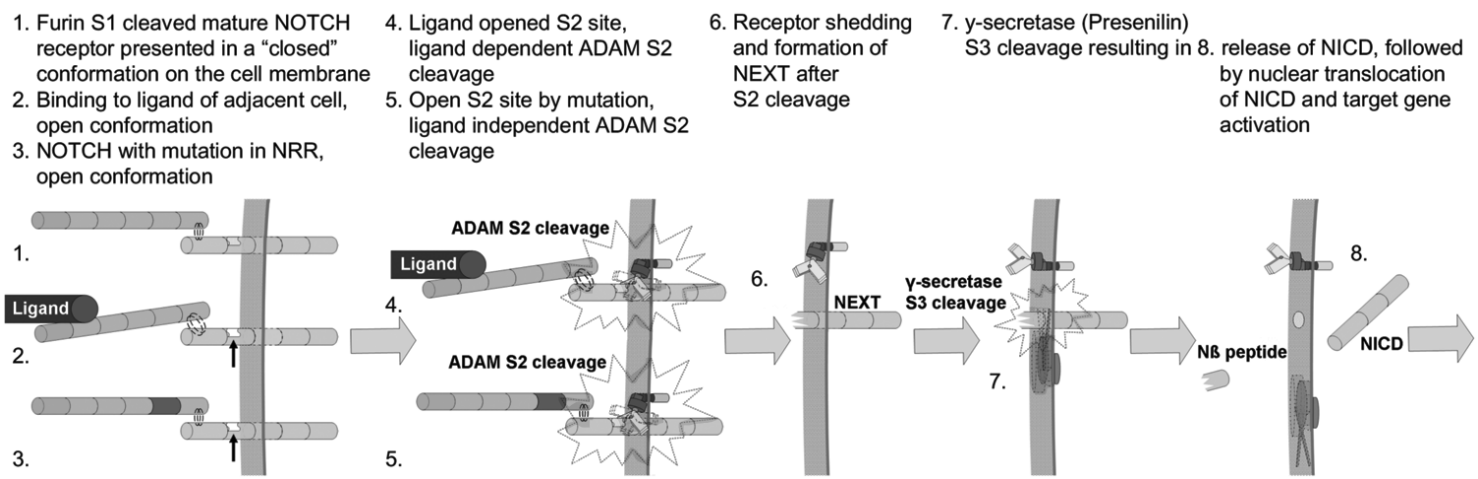


Figure 1. Regulated intramembrane proteolysis activates the Notch pathway. 1) After S1 cleavage in the secretory pathway the mature receptor is presented as a heterodimer at the cell surface in a "closed" conformation. 2) and 3) The notch receptor is activated or "opened" (arrow head) by ligand binding on neighboring cells or by an activating mutation in the NNR. 4) and 5) ADAMs proteases (i.e. ADAM10/Kuzbanian) access and cleave NOTCH at the juxtamembrane S2 site. 6) S2 cleavage results in the membrane tethered NEXT fragment and the notch ectodomain which is transendocytosed in signaling cells. 7) Within the plane of the membrane the NEXT fragment is cleaved at S3 by the aspartyle protease Presenilin which is part of the y-secretase complex. 8) This releases the extracellular Nβ peptide an the intracellular NICD, which translocates to the nucleus to activate target genes.

cleave Type I transmembrane proteins whereas SPP and S2P cleave Type II substrates. A common but not exclusive feature of RIP is that intramembrane proteolysis precedes ligand binding and ectodomain shedding[11]. Cleavage by Rhomboids is an exception and does not require juxtamembrane cleavage prior to intramembranous cleavage. Here we will review our current understanding of how a membrane bound family of metalloproteinases regulate ectodomain shedding and RIP of the NOTCH signaling pathway under physiological and pathological conditions and how this knowledge may be applied for therapeutic intervention.

NOTCH SIGNALING

The Notch pathway is a highly conserved signaling cascade in multicellular eukaryotes and controls spatial patterning, morphogenesis and homeostasis in embryonic and adult tissues.[12,13] Notch proteins orchestrate tissue homeostasis through receptor ligand interactions on adjacent cells.[14,15] Disruption of this homeostatic control by a deregulated Notch cascade underlies cancer formation in several organs.[15-17] Notch receptors (N1 to N4) and ligands (i.e., Delta, Jagged) are Type I transmembrane glycoproteins that transduce signals by binding to membrane bound ligands on adjacent cells. Most if not all Notch functions reported require RIP and constitute the cleavage-dependent or canonical Notch signaling pathway. Upon ligand binding, Notch receptors undergo two successive proteolytic cleavages: an ectodomain cleavage followed by intramembrane proteolysis by γ-secretase[18]. This process releases the Notch intracellular domain (NICD), which translocates to the nucleus and binds CBF1/ Suppressor of Hairless/Lag-1 or CSL (RBP-Jκ in mice) to activate its target genes.[19-23] In the absence of ligand the Notch juxtamembrane localized heterodimerization domain (HD) inhibits extracellular proteolysis and activation.[24,25] The Notch cascade is deregulated in many human cancers and oncogenic mutations in Notch1 are frequently found in human T-cell leukemia that map to the HD and PEST domain,[26] making Notch proteolysis an attractive therapeutic target (Fig. 1).

In *Drosophila melanogaster*, a single prototypical Notch protein is found and two ligands Delta and Serrate (Jagged in mammals). In the nematode *Caenorhabditis elegans*, several Delta-Jagged type ligands (LAG-2/Apx2) are found and two Notch-like receptors, Lin12 and Glp-127. In mammals four Notch genes (Notch1-4) and five ligands: Jagged1-2, Delta-like ((Dll) 1, 3 and 4) are known. These ligands are collectively called DSL ligands (**D**elta and **S**errate/ in *Drosophila* and **L**AG-2 in *C. elegans*).[8] Notch Ligand receptor interactions are regulated by O-linked glysolation/fucosylation of EGF-like repeats in Notch receptor and DSL ligands by Fringe proteins during ER-Golgi transport.[28-30] Glycosylated Notch receptors have higher affinity for Delta than for Jagged type ligands.[31] Physiological Notch activation is triggered by ligand binding and governed by RIP. Most if not all function of Notch require proteolysis.[32,33]

NOTCH PROTEOLYSIS SITE-1 (S1, FURIN, SERINE PROTEASE)

During maturation in the *trans*-Golgi network, Notch precursors are first cleaved at Site-1 (S1) by Furin-like convertase producing a heterodimeric Type I receptor with the Notch extracellular domain (NECD) noncovalently bound to a transmembrane

/intracellular fragment (TMIC).[34-36] Whereas in mammals most canonical (CSL dependent) Notch1 signaling requires furin there are exceptions.[37] S1 cleavage of dNotch is infrequent and not required in flies.[38] Whereas mammalian Notch1 receptors are dependent on furin for proper cell surface expression furin cleavage appears dispensable for Notch2 trafficking and signaling. No information is available on the requirement of furin for Notch3 and Notch4 signaling although they are likely processed in a similar manner.[39] In the absence of ligand mature Notch receptors are held into an inactive "proteolysis resistant" or closed state because the Negative Regulatory Region (NRR) composed of the HD domain and the globular Lin12/Notch repeats (LNR) inhibits Notch activation.[34,40,41] Ligand binding to Notch receptors unfolds the NRR permitting cleavage by a metalloprotease at a site close to the membrane (S2) (Fig. 1).[24,42] Another model involves transendocytosis of NECD upon ligand binding and dissociation of NECD and TMIC heterodimer followed by S2.[43] The latter model however is not consistent with the lack of furin cleavage of dNotch in flies[38] and the activity of ligand–independent gain of function mutations in the NRR although some ligand-requirement still exists.[24,44,45] Not unexpectedly cancer-causing mutations localize to the HD in almost 50% of sporadic human T-ALL causing increased NOTCH1 cleavage and activity.[26,44]

SITE-2 (S2, ADAM10, METALLOPROTEASE)

The S2 cleavage occurs close to the proximal juxtamembrane stalk of the extracellular domain and leads to the shedding of most of the NECD. S2 cleavage is performed by a zinc-dependent metalloprotease and occurs in response to ligand binding that induces a conformational change.[46-48] The membrane-bound cleaved form is termed Notch Extracellular Truncation (NEXT) and is a rate-limiting substrate for the intramembrane cleaving aspartyl protease Presenilin. S2 cleaved Notch fragments are extremely short-lived and can only be observed when blocking γ-secretase processing.[25,48]

SITE-3 (S3, PRESENILIN, ASPARTYL PROTEASE)

Following S2 cleavage, NEXT proteins are cleaved within their transmembrane domain by the GxGD aspartyl protease Presenilin (S3), part of a multi-protein complex termed γ-secretase, releasing the Notch Intracellular Domain (NICD) and the juxtamembranous Nβ peptide.[18] NICD translocates to the nucleus where it binds to the DNA-bound protein CSL together with co-activator proteins Mastermind, leading to target gene activation.[49] The noncanonical signaling pathway that does not rely on CSL (in mice RBP-Jκ), is less well understood and therefore not further discussed here.[50] There is very little evidence for a RIP independent Notch pathway activation. Excellent and more comprehensive reviews on the Notch activation cascade have appeared elsewhere.[8] Here we will summarize our current understanding on the role of ADAM metalloproteases in the activation of Notch signaling cascade and how this may be exploited for therapeutic intervention in diseases with aberrant Notch signaling.

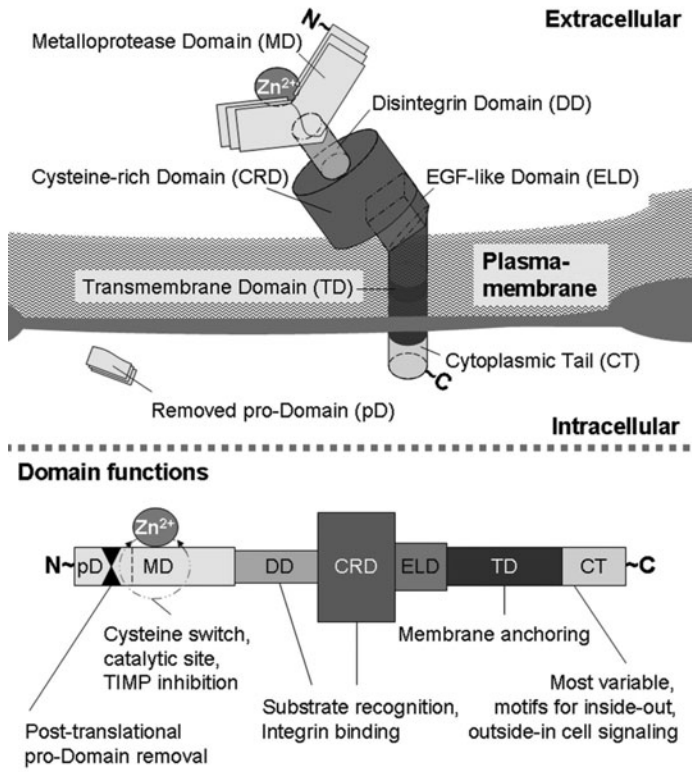


Figure 2. ADAMs structure and function. Upper panel: schematic illustration of an ADAM with extra- and intracellular domains anchored in the plasma membrane. Typical domains from amino- (N~) to carboxy terminus (~C) are: the pro-Domain (pD) shown after removal during maturation of the ADAM, the Metalloprotease Domain (MD) with a zince atome (Zn^{2+}) marking the catalytic site, the Disintegrin Domain (DD), the Cysteine-rich Domain (CRD), the EGF-like Domain (ELD), the Transmembrane Domain (TD) and the Cytoplasmic Tail (CT). Lower panel: ADAM domains and their function. For a detailed description see text. From N~ to ~C: the pD interacts with Zn^{2+} through a cysteine residue at the catalytic stie, called the "cysteine switch." The catalytic site is activated by removal of the pD. ADAM activity can be inhibited by TIMPs. THe MP holds the Zn^{2+} for proteolytic processing of substrates. The DD and ELD recognize substrates and can bind to integrins. The ELD is indispensible but has no known function. The TD anchors the ADAM in the plasma-membrane. The cytoplasmic tail is most variable among ADAMs and has cell signaling motifs such as phosphorylation sites.

THE ADAM FAMILY OF PROTEASES

Disintegrin Metalloproteases (ADAMs) are a distinct class of metalloproteases that have both protease and adhesion domains and play key roles in cell-cell and cell-matrix interactions in diverse cell biological processes such as cell adhesion, fertilization and cytokine and growth factor receptor signaling. ADAMs have a broad tissue expression and are mainly regulated at the posttranslational level. They function as signaling scissors regulating the activity of transmembrane signaling molecules and release soluble ectodomains with an altered location and function.[51,52] In total 40 ADAM family members have been identified in the animal kingdom. Annotated ADAMS of different species

can be found at the White Laboratory (web site 1). Currently in humans 22 ADAM genes are known while in mice 34 ADAM genes have been identified.[53] ADAMs are membrane bound zinc-dependent metalloprotease that belong to the class of astacin/adamalysin family (MEROPS, web site 2).[54] The adamalysins subfamily is comprised of snake venom metalloproteases (SVMP), the membrane bound ADAM and secreted ADAM-TS family. The latter can be structurally distinguished from ADAMs by their various numbers of thrombospondin-like (TS) motifs.[55] ADAM proteins are multidomain proteins that consist of several conserved domains. The amino terminus is composed of a pro-domain followed by the catalytic domain, a disintegrin domain, a cysteine-rich domain containing epidermal-growth factor (EGF)-like repeats, transmembrane domain, and cytoplasmic tail (Fig. 2). The prodomain keeps the protease in a latent state and is clipped off by proprotein convertases (furin) during maturation in the secretory pathway. Some ADAMs lack the furin cleavage site and are activated by autocatalysis.[56,57] The metalloprotease domain encodes the catalytic core sequence **H**EXX**H**XXGXX(**H**/D),[58] that is conserved among ADAM members and comprised of three histidine (**H**) residues that act as a ligand for the catalytic zinc atom (Zn^{2+}) complexed with the O_2 atom from a H_2O molecule. During catalysis, the Zn^{2+} promotes nucleophilic attack on the carbonyl carbon by the oxygen atom of a water molecule at the active site. The conserved glutamate (E) facilitates this reaction by extracting a proton from the attacking water molecule. Zn^{2+} chelating drugs such as hydroxamate-type inhibitors can inhibit most ADAMs although these are mostly broad-spectrum inhibitors.[59] An intramolecular complex can be formed through a cysteine residue in the prodomain and the Zn^{2+} in the catalytic domain keeping the ADAMs catalytic domain inactive, this is called the cysteine switch mechanism.[60,61] For some ADAMs the disintegrin domain (web link 3, PF00200) acts as a receptor antagonist and blocks integrin binding and platelet aggregation.[51] The cysteine-rich domain (web link 3, PF08516) is thought to complement the disintegrin domain in substrate binding. For example the disintegrin and cysteine-rich domains of ADAM13 bind to both integrin receptors and to fibronectin.[62] Besides binding of other proteins on the cell surface there is no evidence that supports another function of the cysteine-rich domain. ADAM proteins differ mostly in their cytoplasmic tails, which are involved in inside-out regulation of metalloprotease activity, outside-in regulation of cell signaling, maturation and subcellular localization and are highly variable in both length and sequence. There are several common motifs including a binding site for SH3-domain containing proteins and putative phosphorylation sites (Fig. 2).[53] In mammals, many ADAMs are expressed exclusively in the male gonads, including ADAM2, 7, 18, 20, 21, 29, and 30. The ADAM8, 9, 10, 11, 12, 15, 17, 19, 22, 23, 28, and 33 have known catalytic activity and a more widespread expression patterns. ADAM-deficient mice have been generated by many groups which are discussed elsewhere.[63] Loss of ADAM2 and 3 both lead to male sterility[64,65] while ADAM9 deficient mice do not show any obvious pathology despite its ubiquitous expression.[66] Moreover, mice that are deficient for ADAM 9, 12 and 15 (Meltrin KO) are viable and fertile and resembled wild-type mice with no defects in shedding of EGFR ligands.[67] This suggests a high functional redundancy between members of the ADAMs family (web link 4).[68] The Tissue Inhibitors of Metalloproteases or TIMPs are a family of highly homologous proteins that are the major natural inhibitors of ADAMs.[69] There are 4 TIMPs and all have been found to have some activity against one or more ADAMs and act in a 1:1 stochiometry. For example ADAM10 and ADAM17 are both inhibited by TIMP3[70,71] and mice lacking *Timp3* have an impaired inflammatory response due to deregulated TACE/ADAM17 activity.[72] In most cases the physiological

relevance of TIMPs on ADAMs remains less clear and requires more study.[52,68] There is ample experimental evidence from flies, worms, and mammals that implicate the ADAM sheddases in the direct cleavage and activation of Notch signaling pathway.

NOTCH RECEPTOR CLEAVAGE BY ADAMs

D. melanogaster express a single Notch protein, and a total of five ADAM metalloproteases.[73] There are two homolog's for ADAM10, Kuz and Kuzbanian-like (Kul) and for ADAM12, DMeltrin and mind-meld (Mmd) and one for tumor necrosis a (TNFa) converting enzyme (TACE)/ADAM17, dTACE.[73-75] The nematode *C. elegans* has two Notch homologs LIN-12 and Glp-1.[27] In worms two ADAMs have been reported to regulate LIN-12/Glp-1/Notch signaling, SUP-17/ADAM10 and ADM-4/ADAM17, respectively.[76]

Genetic studies in *D. melanogaster* and *C. elegans* have demonstrated that ADAM10 (Kuz)/Sup-17 plays a critical role in Notch/LIN-12 signaling *upstream* of S3 γ-secretase cleavage.[77-79] Early fly studies had suggested involvement of Kuz in Notch signaling because of the similarity of the Kuz-mutant phenotype with Notch pathway loss of function phenotypes, caused by defects in lateral inhibition leading to central nervous system hyperplasia and a multiple bristle phenotype.[80] Maternal and zygotic Kuz mutant flies have a more severe phenotype than Notch mutant flies suggesting that Kuz has other substrates as well. For instance, Kuzbanian plays an important role in neuronal guidance by cleavage of Eph/Ephrin receptors.[81-83] Sotillos and Pan provided the first comprehensive analysis on the role of Kuz in the regulation of Notch receptor signaling in flies. Both studies revealed defects in *Kuz*-mutant flies in tissues whose fate is determined by canonical Notch activity.[77, 78] Sotillos cloned the *Kuz* gene from a P-element insertion in mutant flies and demonstrated, by epistatic analysis were, it functions in a dose-dependent manner upstream of S3/NICD cleaved Notch. Notch ligand dependent hyperactive alleles (*Abruptex*) are less active in the absence of *Kuz* whilst NICD rescues partly *Kuz* phenotypes. Altogether these studies demonstrated that Kuz acts cell autonomously in the signal-receiving (Notch expressing) cell, downstream of ligand binding but upstream of S3/NICD cleavage. Since several of the *Kuz* phenotypes seemed less severe than the Notch phenotypes alternative ways for Notch activation must exist.[77] Pan and Rubin demonstrated that *Kuz* phenotypes in flies and frogs could be rescued by wild-type mammalian ADAM10 expression but not by catalytic-dead mutants demonstrating a requirement for proteolytic activity. Similar findings were reported by Wen and colleagues who showed that SUP-17/Kuz phenotypes mimicked Notch/LIN-12 phenotypes in worm.[79] Several groups proposed that Kuz was the protease responsible for furin cleavage (S1) of Notch.[35,78,79] It was only after studies in mammalian cells that demonstrated that the ectodomain cleavage of Notch1 receptor was regulated by ligand binding and is distinct from furin/S1 cleavage during receptor maturation.[48] This cleavage was coined S2 and sensitive to inhibitors of metalloproteases and not to furin inhibitors and occurred between the nonconserved residues Ala1710 and Val1711 (Fig. 3).[47, 48] This finding was later confirmed on endogenous Notch1 signaling as well.[25] By biochemical purification ADAM17/TACE was shown to be required for Notch1 cleavage at Val1711 in vitro excluding Kuz/ADAM10.[47] Also in ligand independent Notch signaling (using constitutively active receptors lacking the EGF-repeat and NRR domains) these proteins still underwent furin/S1 cleavage,[34] which occurred in the absence of Kuz/ADAM10.[48] Lieber et al., presented the first evidence

for a direct role of Kuz in the S2 extracellular cleavage of Notch.[74] Kuz was found to bind Notch directly and induce extracellular cleavage at S2 functioning downstream of ligand binding and upstream of Presenilin S3/NICD cleavage. In the absence of Kuz both S2 and S3 cleavage were impaired consistent with the sequential cleavage model.[48] Whereas in mammalian cells ligand independent Notch molecules function independently of Kuz[48] in flies Kuz was absolutely required for S2. Interestingly, in flies exogenous ADAM17/TACE can partly rescue the *Kuz* cleavage defect in vitro.[74]

The discrepancies between the Kuz requirement in flies and Tace requirement in mammalian cells can now be reconciled by recent work. Both the Weinmaster and Vooijs labs conclusively demonstrated that ADAM10 has an essential role in ligand dependent Notch1 signaling but that ADAM17/TACE or other ADAM proteases may function redundantly with Kuz/ADAM10 particularly in ligand-independent Notch signaling proteins such as those found mutated in cancer.[25,45] In vitro and in flies both Kuz/ADAM10 and TACE are sufficient to cleave Notch in the absence of ligand, a process that may be important in tumors overexpressing ADAM proteins[74,84,85] and van Tetering et al., in preparation). Also in worms inactivation of either SUP-17/ADAM10 or ADM-4/ADAM17 has no dramatic effect on LIN-12/Glp-1/Notch signaling however their combined inactivation produces a Notch loss of function phenotype suggesting that these proteases act redundant. Many tissues in SUP-17/ADM-4 mutants however appeared normal suggesting that Notch activation in other tissues requires the action of other unknown (ADAM-like) proteases in *C.elegans* to regulate LIN-12/Glp-1 activity of which there are several.[76]

Mice lacking *Notch1* are embryonic lethal at E9.5 with severe defects in haematopoiesis and neurogenesis.[33,86,87] Whereas ADAM17/TACE copurifies with Notch1 processing activity in HeLa cells and can cleave Notch1 in vitro, *Adam17/Tace*-deficient mice or flies do not phenocopy the Notch-deficient phenotype suggesting that it is either redundant with another protease or has a more spatial or temporal restricted role in Notch signaling.[74,88] TACE may have more tissue-restricted roles in regulating Notch1 cleavage however, for example during differentiation of monocyte progenitors into macrophages.[47] In line with the *kuz* phenotypes in flies, mice lacking Adam10 die at E9.5 with reduced neuronal *Hes*5 expression, a Notch target gene, resembling *Notch1*-null embryos.[89] Moreover, T-cell-specific deletion/disruption of *Adam*10 in vivo phenocopied the *Notch1* null phenotype during thymocyte development.[90-92] Recently by using ADAM10 silencing by siRNA or *Adam*10 KO fibroblasts it was finally demonstrated that ADAM10 is required for ligand-dependent endogenous Notch1 signaling and that it executes S2 cleavage at Val1711.[25,45] In contrast to flies overexpression of ADAM17 could not compensate for ADAM10 loss.[45] This finding was recently confirmed by the analysis of brain-specific *Adam*10 deletion showing a severe defect in Notch cleavage and signaling in mice.[93] Some residual S2/S3 cleavage could be detected in the absence of Adam10 indicating a different protease is capable of cleaving Notch1 as well.[93]

ADAM10 however may also be involved in ligand independent signaling and appears to act redundant with TACE/ADAM17 in the activation of T-ALL derived Notch proteins.[25,45] The Meltrins ADAM9, ADAM12, and ADAM15, all have been implicated in Notch signaling directly or indirectly by activating ADAM10[94,95] however appear not to be required for Notch1 proteolysis.[25] As mentioned these findings were somewhat unanticipated because earlier no defects were observed in Notch cleavage.[48,89] This can now be reconciled by the demonstration that pathological (ligand independent) and physiological Notch signaling may engage distinct sheddases.[45]

A tantalizing new finding is the involvement of the nonmembrane bound Matrix Metalloproteinase-7 (MMP-7) in Notch1 activation. Sawey and colleagues convincingly demonstrated that in pancreatic explants derived from *Mmp7*-/- mice acinar transdifferentiation -a Notch1 dependent process- is defective due to impaired Notch cleavage.[96] Direct proof for the involvement of Mmp7 in ligand-independent Notch1 cleavage at S2 awaits further analysis but would be remarkable given the "protected" nature of the S2 scissile bond deep within the juxtamembrane LNR module.[24] This study further illustrates the diversity of proteolytic cascade involved in Notch activation of particular relevance to pancreatic cancer but may apply to other cancer types as well. Until now there is little insight into the proteases and mechanism involved in shedding of Notch2, Notch3 and Notch4. ADAM10 has been implicated in marginal zone B-cell development; a Notch2 dependent process however no direct effect on Notch2 cleavage was shown.[97] It is likely their activation follows a similar paradigm as for Notch1.

NOTCH LIGAND CLEAVAGE BY ADAMS

A series of papers in 2003 reported that mammalian Notch ligands are also subject to RIP. Delta and Jagged are both cleaved by multiple ADAMs including ADAM10 ADAM17/TACE, ADAM12 and ADAM9 followed by γ-secretase cleavage.[94,98-101]. These findings support earlier work on the involvement of Delta cleavage in vivo in flies.[102, 103] Kuz is both necessary and sufficient to induce Dl cleavage and sensitive to metalloprotease inhibitors.[102] Although the exact nature and role of ligand cleavage in Notch receptor signaling remains unresolved, a role for Kuz in Notch ligand cleavage would be consistent with a role for soluble ligands in Notch/LIN-12 activation in worms.[104] However in flies and mammals soluble Notch ligands are inactive or act antagonistically/competitively with membrane bound forms.[105,106] Furthermore, Delta intracellular domain is required for some ligand mediated Notch activation in flies and mammals and promotes ligand multimerization thought to be necessary for efficient Notch activation.[107,108]

Kuz cleavage of Delta may also explain the non-cell autonomous phenotypes observed for Kuz in fliesm[80] although only *cis*-cleavage by Kuz has been shown.[109] Delta cleavage would be consistent with models of ligand transendocytosis in receiving cells.[101,109] Similar to Notch1 the P-P1' cleavage sites are conserved between mammals but mutation of S2 cleavage sites does not prevent cleavage whereas pharmacological and genetic protease inhibition does.[25,101] Whereas Notch1 S2 cleavage occurs at the cell surface (Fig. 1)[25] the subcellular location of ligand cleavage has not been determined but may—depend on and—occur during endocytosis.[110,111] A comprehensive review on ligand cleavage has been published elsewhere.[112]

Kuz has also been postulated to act in *cis*-inhibition whereby ligands in Notch receptor expressing cells prevent Notch receptors binding to ligands and signaling in *trans* to adjacent cells.[113,114] Thus a general picture emerges whereby RIP regulates the activities of Notch receptors and ligands to achieve a delicate balance and directionality of signaling needed for context dependent fine-tuning including in the maintenance of stem cells in the *Drosophila* mid gut [115] as well as in the mammalian epidermis.[116] An interesting twist to the story is emerging by the identification of Kul (Kuz-like) the Kuzbanian homolog in flies. Interestingly no Kul homolog has been identified in mammals. Kul protease appears to be critical to maintain signaling directionality during lateral specification in flies.[73] An important role for Kul has been demonstrated in wing margin specification where Kuz

had already been demonstrated to be dispensable for Dl signaling/processing.[75] Whether Kul's role extends to other invertebrate tissues as well is not known and why flies have evolved to have an additional protease for Delta processing is intriguing.

NOTCH S2-PROTECTION FROM RIP

Under physiological conditions the Notch receptor requires binding of DSL ligands from adjacent cells in trans to EGF repeats 10-12 on Notch expressing cells.[117] In the absence of ligand the Notch juxtamembrane region or negative control region (NRR) comprising the LNR and HD domain (Fig. 1) acts to inhibit Notch proteolysis.[34,40,41,118] The role of ligand binding and endocytosis in triggering Notch receptor activation by RIP has emerged as a key step in the Notch activation cascade.[46,48] Both genetic and biochemical studies supported a model in which receptor oligomerization would regulate receptor proteolysis.[40,48] Notch receptors at the cell surface are mostly in their monomeric form with or without ligand. Although Notch proteins can hetero- and homodimerize mediated by the EGF repeats this appears not to regulate shedding of the extracellular domain, since ligand independent active and inactive proteins do not differ in oligomerization.[119] Dimerization of the Notch extracellular domain was recently confirmed by electron microscopy.[120] It will be interesting to see whether Notch receptors carrying T-ALL mutations have altered dimerization properties. Although from mammalian studies it appears unlikely that dimerization regulates Notch receptor function[119] in *Drosophila*, where furin cleavage is infrequent, dimerization of Notch receptors may play a more prominent role in regulating activity.

The finding that in about 25% of human T-ALL cases mutations are found in the NRR that cause destabilization, ligand-independent Notch cleavage and activation underscores the central importance NRR unfolding in the activation cascade.[26,44,121] X-ray structural analysis and epitope-specific antibodies have helped us understand how this activation occurs. Ligand binding to Notch dissolves the "closed" NRR structure leading to access to the Val1711 scissile bond.[24,25] Importantly both physiological and ligand independent Notch molecules are cleaved at the same position. These results suggest that conformational changes induced in the extracellular domain by DSL binding or by cancerous mutation are similar but engage different sheddases.[25] Further support for this comes from the analysis of Notch proteins with heterologous (CD4ΔIg) extracellular domains.[48] These constitutively active proteins are also cleaved at Val1711[119] (and Vooijs et al., unpublished).

Currently two models prevail that explain access to S2. Both rely on the force generated between membrane bound ligands and Notch receptors on adjacent cells to liberate the NECD that has been correlated with the extent of adhesion between receptor and ligands.[122] One based on X-ray structural analysis predicts that destabilization of the NRR "lifts" the LNR modules to allow S2 protease access.[24] The other proposes that ligand binding leads to physical dissociation of the noncovalently held NRR and after which S2 proteolysis occurs.[43] It is clear that for cancer prone ligand-independent Notch receptors, NRR relaxation is sufficient to induce S2 as opposed to ligand dependent signaling whereby ligand endocytosis may be needed to pull apart the noncovalent heterodimer. Irrespective of both models the S2 cleavage remains a limiting step for S3 proteolysis. More detailed analysis is needed and X-ray structures

from mutated Notch receptors are eagerly awaited. How then is the specificity of S2 proteolysis regulated?

SUBSTRATE REQUIREMENTS FOR S2

Regulation of S2 proteolysis by ADAMs may occur at transcriptional levels by gene expression and alternative splicing, subcellular distribution and posttranslational modification. One of these is prodomain removal by furin in the secretory pathway, which ensures cell surface expression and activity. For example furin and PC7 are critical for ADAM10 maturation and cell surface activity.[123] Thus defects in furin may not only affect Notch1 processing at S1 but also S2 processing by ADAM10.[25]

To date little is known on what determines substrate specificity. Overall cleavage sites are highly variable and a clear consensus sequence is lacking, the secondary structure and the distance of the juxtamembrane stalk (for Notch1, APP, TNFα and EGF ligands are all located within 20 amino-acids proximal to the TMD) seem to be of critical importance for efficient substrate recognition.[53,124] Elegant experiment from Arribas demonstrated that swapping the juxtamembrane domains from ADAM permissive substrates TGFa and APP with a substrate normally not shed, could convert this chimeric protein into an efficient ADAM substrate.[125] Early work from the Struhl and Kopan labs[48,126] had already demonstrated that the extracellular domain of Notch inhibited S2 cleavage in vitro and in vivo. Using heterologous extracellular domains trimming of the extracellular domain of Notch increased signaling activity in vivo. Engineered dimerization/oligomerization of the extracellular domain also inhibited S2 and S3 cleavage of Notch.[48] These experiments established for the first time that S2 was an intermediate step between S1 and S3 and tightly (negatively) regulated by NECD. Although γ-secretase binds to membrane bound Notch during maturation in the secretory pathway the generation of a free N-terminus close to the transmembrane domain is required to become a RIP substrate. Shah provided evidence that Nicastrin an essential component of the γ-secretase activity functions as a docking receptor to haul S2 cleaved Notch into the RIP-S3 processing machinery.[127]

Interestingly like for S3 Presenilin cleavage of Notch1 the substrate requirements for S2 seem to be independent of the intracellular domain. Furthermore, NEXT-like Notch1 proteins (N1ΔE) that are highly active are still S2 cleaved. Cleavage at Val1711 can be blocked by pharmacological or genetic ADAM10 inhibition but has no consequence for γ-secretase cleavage or transcriptional activity. This shows that, at least with truncated artificial substrates like N1ΔE, ADAM proteins will produce Val1711 even with only several residues at the C terminal- cleavage site and further question the substrate requirements for S2 cleavage.[25] Moreover, these data suggest that cleavage by ADAM10, under some conditions, may not require binding to Notch. This opens up the hypothesis that the S2/S3 RIP complex is already pre-associated at the cell surface and that the substrate and protease transmembrane domains may interact in the lipid bilayer.

By using randomized peptides libraries the in vitro cleavages specificities of TACE/ADAM17 versus ADAM10 has revealed distinct amino-acid preferences.[128] The most important difference maps to the P1' position of the substrate where TACE is selective for smaller aliphatic residues and ADAM10 can accommodate aromatic amino acids. Whereas the P1-P1′ (Ala-Val) amino acids in the mNotch1 S2 cleavage

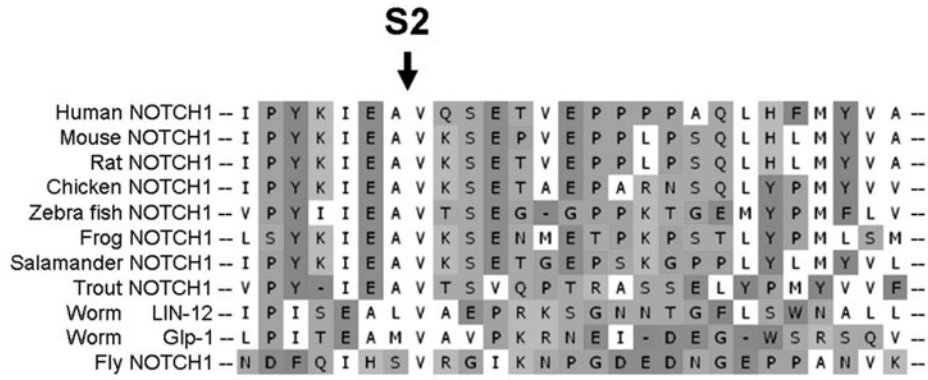


Figure 3. Conservation of the S2 ADAM cleavage site. Partial alignment of NOTCH1 proteins from different species, with the arrow indicating the S2 cleavage site. Image of alignment was constructed using the program UTOPIA. Aminoacides, in green: Asparagine (N), Glutamine (Q), Serine (S) and Threonine (T); in red: Aspartic acid (D) and Glutamic acid (E); in purple: Phenylalanine (F) and Tyrosine (Y); in blue: Histidine (H), Lysine (K) and Arginine (R); in orange: Glycine (G) and Proline (P); in white: Alanine (A), Isoleucine (I), Leucine (L), Methionine (M) and Valine (V).

site are conserved Notch1 cleavage at Val1711 still has to be demonstrated in other species. Remarkably, whereas the P1-P1′ A-V motif in Notch1 is common in multiple TACE substrates it is unique among known ADAM10 substrates.[128] In view of this and the finding that recombinant murine Notch1 proteins/peptides are preferred by ADAM17/TACE[47] whereas in vivo mNotch1 proteins require ADAM10 these findings suggest that protease activity in vitro may not always be extrapolated.[25] In mammals the S2 cleavage site of Notch1 **Y**x**IEA**-**V**x**SE** is highly conserved between species between Notch proteins of different species only AV is conserved (Fig. 3). Based on this analysis NOTCH receptors 2, 3 and 4 are also predicted to be substrates for TACE, but no results have been reported on this yet.

Mutation of the Notch1 S2 cleavage site does not abolish cleavage but generates a novel epitope, which has not been determined.[25] P1- mutants of other ADAM10 substrates such as lysine (P1-K|L-P1′) in APP also does not abolish cleavage.[129] These findings further support the notion that structural alterations induced by mutation or ligand-binding expose scissile residues that enable access of the sheddase and cleavage. Direct confirmation for this has come from the analysis of T-ALL mutations that affect the S2 position as well as direct mutation of the S2 cleavage site, which produces a gain of function protein.[25,130]

ADAM10 and TACE/ADAM17 function in multiple RIP processes such as cleavage of the extracellular domain of Alzheimer's precursor protein (APP), TNFa and EGF ligands among many other Type I integral membrane proteins.[131] ADAM10 has up to 40 substrates including Type II membrane proteins that are SPP substrates.[132] Although ligand-independent activity induced by ADAM expression has been described this is probably not a physiological mechanism.[84] A common feature of ectodomain shedding is constitutive versus regulated shedding. For example ADAM10 is required

for constitutive α-secretase cleavage of APP[133] whereas both ADAM17/TACE and ADAM10 are involved in PMA-stimulated (regulated) shedding.[134,135] Thus distinct proteases are involved in constitutive versus regulated cleavage of RIP substrates Notch and APP. Whereas Notch gain of function mutations are constitutively cleaved, this cleavage can be further induced by PMA, the mercuric compound APMA and well as by EDTA, the latter by disrupting the noncovalently associated heterodimer.[25] If during constitutive and regulated cleavages of Notch different proteases are recruited that cleave Notch at different positions has to be determined. The only evidence for this so far stem from the analysis of Adam17/Tace requirement to block PMA stimulated differentiation of monocyte precursors into macrophages a process that depends on Notch-Jagged signaling.[47] It should be noted that it is not clear whether PMA/APMA regulated shedding of Notch receptors has any physiological role.

LOCATION OF S2 CLEAVAGE

The best characterized substrates for ADAM proteases are membrane bound signaling molecules such EGF ligands, TNFα, APP, Ephrins and Notch[136] Most ADAM proteases are produced as inactive zymogens and activated by prodomain removal in the secretory pathway. Prodomain removal is necessary for proper cell surface expression. The α-secretase cleavage of APP by ADAM10 predominantly occurs at the plasma membrane,[129] but also in the *trans*-Golgi network upon stimulation with PMA, where it may compete with β-secretase cleavage.[137] Intracellular cleavage of APP induced by PMA is consistent with the perinuclear expression of TACE.[137,138] Using S2-epitope specific antibodies van Tetering showed by immunofluorescence and biochemically that Notch S2 cleavage by ADAM10 predominantly occurred at the cell surface. Interestingly this analysis also demonstrated intracellular vesicles that were decorated with the S2 antibody. As this analysis was performed in the presence of γ-secretase inhibitor this indicated that S2 cleaved Notch from the cell surface may be internalized for further processing. At present the identity and fate of these S2-positive vesicles is not known but of great interest. Outstanding questions are whether these vesicles contain γ-secretase competent Notch molecules and whether these are recycled to the plasma membrane or follow other intracellular routes for activation or degradation.[25] Endocytosis is a conserved biological mechanism regulating the location and activity of membrane bound signaling molecules.[139] Also in the activation of Notch signaling, ligand endocytosis plays a critical role presumably to pull apart the NRR to expose the S2 site.[46] Furthermore, asymmetric cell division and unequal distribution of Notch receptors and ligands among daughter cells controls important cell fate decisions in several tissues.[140] There is compelling evidence that demonstrates that receptor endocytosis controls Notch cleavage and activity in signal receiving cells independent of ligand endocytosis in signaling cells in flies and mammals.[26,141-143] Several years ago it was already established that γ-secretase cleavage sites on Notch1 and APP shifted in the more acidic environments of endocytic vesicles. NICD species with different NH2-residues (V, L, S) have different stability and therefore signaling strength, which highlighted an additional level of regulation.[144-146] At least under some conditions receptor endocytosis is required for γ-secretase cleavage.[146-148] From this it is clear that γ-secretase activity occurs both at the plasma membrane as well as in endocytic vesicles. For example the E3 ubiquitin ligase Deltex that directly binds to Notch

promotes signaling independent of ligand via receptor internalization in endocytic compartments. Depending on the cofactors AP-3/HOPS and type of endosome (early vs late) the fate of Notch receptors is degradation or signaling.[143,149] Under conditions of signaling the Notch ectodomain is removed however whether this is as a consequence of ectodomain shedding at S2 (or an alternative position) or non-specific degradation is unknown.[149] Whether similar roles for Deltex can be extended to other tissues and species is not known but ligand-independent Notch activation via Deltex may occur through heterodimer destabilization due to changes in pH/ionic environments in endocytic organelles.[150] Exciting new data is emerging that show that ligand dependent Notch signaling may also occur in multivesicular endosomes so-called Sara endosomes.[151] Whether Sara endosomes present a conserved intracellular Notch signaling node is not known but exiting new insights are expected. The availability of antibodies for S2 cleaved forms of Notch1 was long awaited and may be used to address the origin and fate of S2 activation step in mammalian cells as well.[25]

TARGETING S2 PROTEOLYSIS IN CANCER

In the last decade it has become apparent that deregulation of the Notch signaling pathway is a common event in human cancer. This research was fueled by the discovery that Notch mutations and alterations underlie the pathogenesis of T-cell acute lymphocytic leukemias (T-ALL).[26,152] Missense mutations in the extracellular HD of Notch1 occur in about 25% of all human T-ALL cases[26] and lead to ligand independent activation.[44] Less frequent are insertions that duplicate the S2 site and lead to ligand independent Notch activity.[130] Finally alterations in the endocytic pathway as discussed above may provide another oncogenic pathway for Notch activation. In addition to T-ALL, Notch activation is common in solid cancers as well. For example in breast cancer one of the most frequent malignancies in the western world Notch1 activation is a common event and correlates with poor prognosis.[153-156] There is ample evidence for crosstalk with other frequently deregulated pathways in breast cancer such as RAS/EGFR/HER2[157] activation and Estrogen pathway[158] that are common drug targets for cancer intervention. New data is emerging that activation of Notch3 and Notch4 as well may also be important in breast cancer. In contrast Notch2 activity seems to correlate with better prognosis in breast cancer.[159] Finally, Notch receptor signaling is critical in normal mammary stem cell[160, 161] as well as in tumor initiating cells or breast cancer stem cells.[162] Finally an important new role of Notch signaling is emerging in maintaining normal and tumor angiogenesis (reviewed in ref. 163). The role of Notch signaling in cancers will be discussed in detail elsewhere. Given the widespread role and importance of NOTCH activation in human malignancies therapeutic targeting of Notch may allow for disease control. Most if not all Notch signaling requires S2 and S3 cleavage making Notch proteolysis an attractive drug target. At the same time this provides challenges for cancer drug development, as these drugs will also target physiological Notch activation. Currently 15 clinical trials are underway that evaluate the efficacy of γ-secretase inhibitors (GSIs) as anti-cancer drugs (web link 4). While targeting γ-secretase using has shown encouraging results it has many pitfalls as well. Among these is the lack of specificity and the mechanism based toxicity caused by attenuating physiological Notch function. One of the best-illustrated side-effects is gastrointestinal toxicity caused by precocious secretory differentiation of intestinal epithelial cells limiting its long term use because of intestinal stem cell

depletion.[17,164,165] Both pharmacological and genetic approaches have provided insight into the normal role of Notch in suppressing secretory differentiations and have revealed KLF4 as a key regulator. Significant progress was made by Real and colleagues who demonstrated that combined glucocorticoid and GSI treatment increased anti-leukemic activity in T-ALL by reversing glucocorticoid resistance while suppressing gut toxicity by inhibition of KLF4[166]. Since γ-secretase has many different substrates pleiotropic effects are unavoidable when targeting this enzyme, although modulators have been identified that may show selectivity for specific substrates.[167] The fact that GSI have been studied intensely the past decade a safe-drug has yet to enter clinical practice.[168] Recently, monoclonal antibody based approaches have been developed specifically targeting the Notch NRR that show promising results in preclinical studies.[169-171] Strategies to target the nuclear transcription complex are also promising.[172]

An alternative approach could encompass targeting S2 cleavage: the rate-limiting step in the Notch activation cascade in physiological and pathological conditions.[25] Two approaches not mutually exclusive could be envisaged. One based on targeting the metalloprotease, another based on blocking proteolysis of S2 cleaved Notch. Multiple ADAMs have causal roles in cancer formation and progression but whether these ADAM deregulate Notch activation in these tumors is unknown (reviewed in ref. 173). Despite enormous efforts and investment the implementation of metalloprotease inhibitors in anti-cancer medicine has largely failed.[174] For example, the hydroxamic acid Zn^{2+} chelators GM6001 (Gelardin), Batimastat (BB94) and Marimastat have all failed for clinical application because of undesirable side-effects. Recently progress has been made with the development of new inhibitors more specific for ADAM10 and ADAM17. One of these INCB3619 is designed to target EGFR/HER shedding a frequent and causal alteration in breast and lung cancer.[175-177] Since Notch activity is frequently reported in both cancers it will be interesting to see whether inhibition of Notch shedding occurs and whether this is of therapeutic value.

While investigating the requirement for ADAM proteases on Notch S2 cleavage we discovered that S2 cleavage and transcriptional activity of activated Notch1 could neither be blocked using broad-spectrum metalloprotease inhibitors (GM6001, BB94) nor with more specific ADAM17/TACE or ADAM10 specific inhibitors.[178] Interestingly whereas these inhibitors efficiently blocked Val1711 cleavage residual S2 cleavage occurred at another unknown position, $S2^b$. Thus whereas ligand dependent Notch signaling requires ADAM10, ADAM/MP inhibition in ligand independent signaling does not abrogate activity. The $S2^b$ cleavage is insensitive to broad-spectrum aspartyl, cysteine and serine protease inhibitors as well as to BACE inhibitors. These results strongly point to the existence of additional S2 proteases insensitive to these mechanism- based metalloprotease inhibitors.[25] One likely outcome from these and other studies is that pathological (ligand-independent) and physiological Notch signaling may engage distinct sheddases.[45] While this hypothesis awaits further validation it suggests the possibility of targeting disease-specific Notch proteases while leaving normal Notch signaling intact.

Another potential strategy to block Notch activity in tumors is to prevent conversion from S2 cleaved Notch into a γ-secretase substrate using S2 specific antibodies.[25] Such capping antibodies would be highly specific and could interfere with efficient recruitment of S2 cleaved into the γ-secretase complex. Such antibodies would target receptors and are not hampered by membrane diffusion or cellular uptake. Experimental data suggests that such an approach may be feasible.[127] It is important to note that completely blocking Val1711 pharmacologically however does not abrogate signaling and physiological and pathological Notch signaling both proceed through Val1711 cleavage.

It is clear that many challenges still lie ahead before drugs will be developed that are effective in controlling Notch dependent disease while leaving normal Notch signaling intact.

CONCLUSION

The highly conserved Notch signaling pathway controls developmental patterning and homeostasis in animal tissues. A conserved proteolytic cascade leading to liberation of the Notch intracellular domain and transcription governs the rate-limiting step in Notch activation: ectodomain shedding at S2. Loss of function studies point to a dominant role for ADAM10 in the cleavage and activation of Notch1 after ligand binding. Under malignant conditions shedding of Notch1 receptors is induced by different proteases that eagerly await identification. Exciting biology lies ahead of us in revealing the many facets of NOTCH proteolysis controlling Notch activation in normal and injured tissues providing new insights that may be applied for therapeutic intervention.

WEB LINKS

1. White lab: http://people.virginia.edu/~jw7g/Table_of_the_ADAMs.html.
2. MEROPS Protease database (REF): http://merops.sanger.ac.uk
3. Protein families and Domains: http://pfam.sanger.ac.uk
4. GSI clinical trials: http://clinicaltrials.gov/ct2/results?term=NOTCH+and+CancerGSI clinical trials

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CHAPTER 3

METABOLISM AND TRANSPORTATION PATHWAYS OF GDP-FUCOSE THAT ARE REQUIRED FOR THE *O*-FUCOSYLATION OF NOTCH

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Abstract: Notch is a single-pass transmembrane receptor that mediates the local cell-cell interactions necessary for many cell-fate decisions. The extra cellular domain of Notch contains a tandem array of epidermal growth factor-like (EGF-like) repeats. Some of these EGF-like repeats are *O*-fucosylated by protein *O*-fucosyltransferase 1 (*O*-fut1), which is essential for Notch signaling in *Drosophila* and mouse. This *O*-fucose is further modified by Fringe, a GlcNAc transferase and other glycosyltransferases (*O*-fut1 in *Drosophila* and Pofut1 in mouse), to form an *O*-linked tetrasaccharide, which modulates Notch's selective binding to its ligands and activates Fringe-dependent Notch signaling in some developmental contexts.

INTRODUCTION

The enzymatic reaction of *O*-fut1 and other fucosyltransferases requires GDP-fucose as a fucose donor. Thus, GDP-fucose synthesis is essential for the fucosylations of Notch and the Fringe-dependent activation of Notch signaling. Although GDP-fucose is synthesized in the cytoplasm, *O*-fucosylation occurs in the lumen of the endoplasmic reticulum (ER). Therefore, GDP-fucose must be transported into the ER lumen from the cytoplasm. A GDP-fucose transporter (SLC35C1) is the product of the gene responsible for the human congenital disorder of glycosylation Type IIc (CDG IIc). In *Drosophila*, two GDP-fucose transporters named for their intracellular localization, Golgi GDP-fucose transporter (Gfr) and ER GDP-fucosetransporter (Efr), have been identified. Efr and Gfr function redundantly

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to enable the *O*-fucosylation of Notch EGF-like repeats by *O*-fut1. Thus, two pathways for the nucleotide sugar supply, involving two nucleotide sugar transporters with distinct characteristics and distributions, contribute to the *O*-fucosylation of Notch. A partial reduction in Notch signaling activity may account for the pathology of CDG IIc.

PROPER STRUCTURE OF ITS EXTRACELLULAR DOMAIN IS CRUCIAL FOR THE ACTIVATION OF NOTCH

Cell-cell interactions are essential for the development of multicellular organisms. Notch signaling is mediated by directional cell-cell contacts and regulates various cell-fate specifications.[1] Notch family proteins are single-pass transmembrane receptors with multiple epidermal growth factor(EGF)-like repeats in their extracellular domain.[2,3] These EGF-like repeats serve as the binding sites for the DSL (Delta/Serrate/Lag-2) and DOS (Delta and OSM-11-like protein) domains in the extracellular region of Notch'stransmembrane ligands, e.g., Delta and Serrate in *Drosophila*.[4] As expected from the complex structure of the *Drosophila* Notch extracellular domain, which has 36 EGF-like repeats and other motifs, Notch activation depends on its extracellular domain having the proper structure,[5] which is partly determined by the glycan modification of its EGF-like repeats.

MODIFICATIONS OF *O*-LINKED GLYCANS IN THE EGF-LIKE REPEATS OF NOTCH

Currently, two distinct *O*-glycan modifications in the EGF-like repeats of Notch are known: *O*-fucose and *O*-glucose.[6] The *O*-fucosylation of EGF-like repeats was first identified in urokinases.[7] A comparison of the amino acid sequences of EGF-like repeats that are *O*-fucosylated led to the identification of a consensus sequence, Cys^{2}-X^{4-5}-Ser/Thr-Cys^{3}, in which the Ser or Thr residue is *O*-fucosylated (Fig. 2).[8] Protein *O*-fucosyltransferase-1, an evolutionarily conserved ER residential Type-II transmembrane protein, catalyzes this *O*-fucosylation.[9] The *O*-fucosylation consensus sequence is present in 23 of the 36 EGF-like repeats of *Drosophila* Notch (Fig. 1).

Fringe family proteins are evolutionarily conserved β1, 3*N*-acetylglucosaminyltransferases that add a GlcNAc specifically to the *O*-linked fucose of the Notch EGF-like repeats.[10,11] This modification of EGF-like repeat 12 of mouse Notch1 induces a conformational change.[12] In mammalian cells, Sia-α2, 3-Gal-β1, 4 is further added to the GlcNAc and consequently an *O*-linked tetrasaccharide (Sia-α2, 3-Gal-β1, 4-GlcNAc-β1, 3-Fuc) is formed on these EGF-like repeats (Fig. 1). This *O*-linked tetrasaccharide modification of Notch promotes its binding to Delta-type ligands and suppresses its binding to Serrate-type ligands (Fig. 2).[13] The expression patterns of *fringe* homologs and Notch ligands are highly tissue-specific in severalspecies. Because Fringe family proteins can differentially modify Notch's interactions with its ligands, Notch signaling is often activated along the boundaries of Fringe-expressing and -non-expressing domains.[14] Although the Delta- and Serrate-type ligands also contain EGF-like repeats that are *O*-fucosylated, these modifications have not been found to contribute to the regulation of Notch signaling so far.[8]

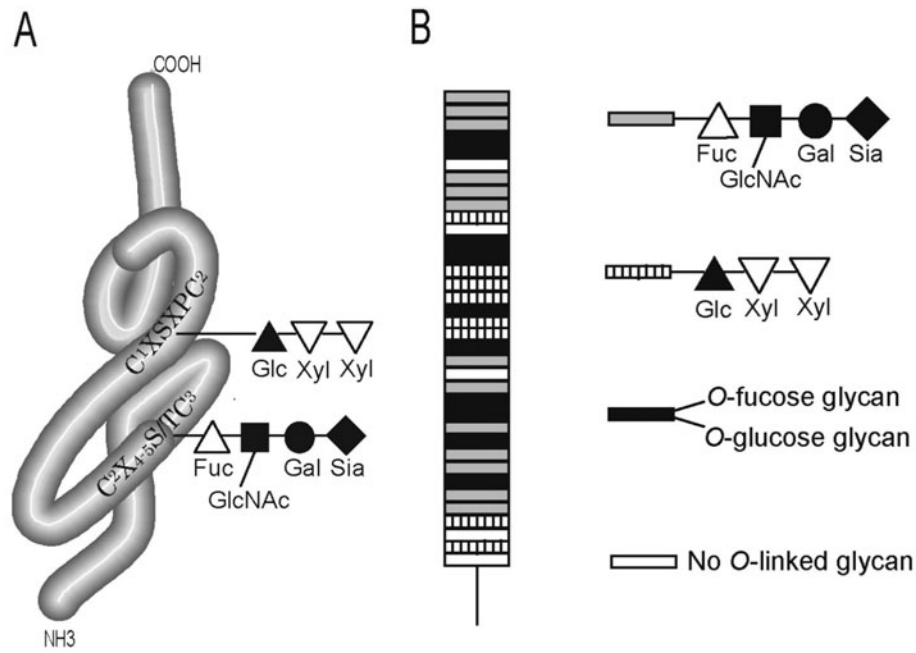


Figure 1. *O*-linked glycan modification sites in the EGF-like repeats of Notch. A) The structure of the EGF-like repeats of Notch is shown schematically. The *O*-linked tetrasaccharide (Sia-α2,3-Gal-β1,4-GlcNAc-β1,3-Fuc) is added to the Ser/Thr residue in the $C^2X_{4-5}S/TC^3$ consensus sequence. The *O*-linked trisaccharide (D-Xyl-alpha1-3-D-Xyl-alpha1-3-D-Glc) is added to the Ser residue in the C^1XSXPC^2 consensus sequence. B) Variety of *O*-linked glycan modifications in the 36 EGF-like repeats of *Drosophila* Notch. EGF-like repeats (indicated by rectangles) that have the consensus sequence(s) for only *O*-fucosylation (gray), only *O*-glucosylation (stripe) and both *O*-fucosylation and *O*-glucosylation (black) are shown. EGF-like repeats without these consensus sequences are indicated by open rectangles.

O-glucose glycan is another sugar modification of Notch EGF-like repeats.[6] The consensus sequence (Cys^1-X-Ser-X-Pro-Cys^2) for this modification is different from that for *O*-fut1[6] andis present in 18 of the 36 EGF-like repeats of *Drosophila* Notch (Fig. 1). Among the EGF-like repeats of mammalian Notch1, one class undergoes only *O*-glucose modification, while another simultaneously undergoes *O*-fucose and *O*-glucose modifications (Fig. 1).[6] *Drosophila rumi* encodes the *O*-glucosyltransferase responsible for the *O*-glucosylation of Notch EGF-like repeats.[15] Two xylose moieties are further added to this *O*-glucose, to form an *O*-linked trisaccharide.[16] *Drosophila* mutant embryos with a disruption in *rumi* show neuronal hyperplasia, which is reminiscent of *Notch* loss-of-function mutants.[15] This neuronal hyperplasia is temperature-sensitive, suggesting that the *O*-glucose glycan modification may be involved in Notch folding.[15] Consistent with this idea, Notch is not localized to the surface of *Rumi* mutant cells in *Drosophila*, but is instead mislocalized to intracellular vesicles.[15] However, the roles of this *O*-glucose glycan modification in vertebrates are not known.

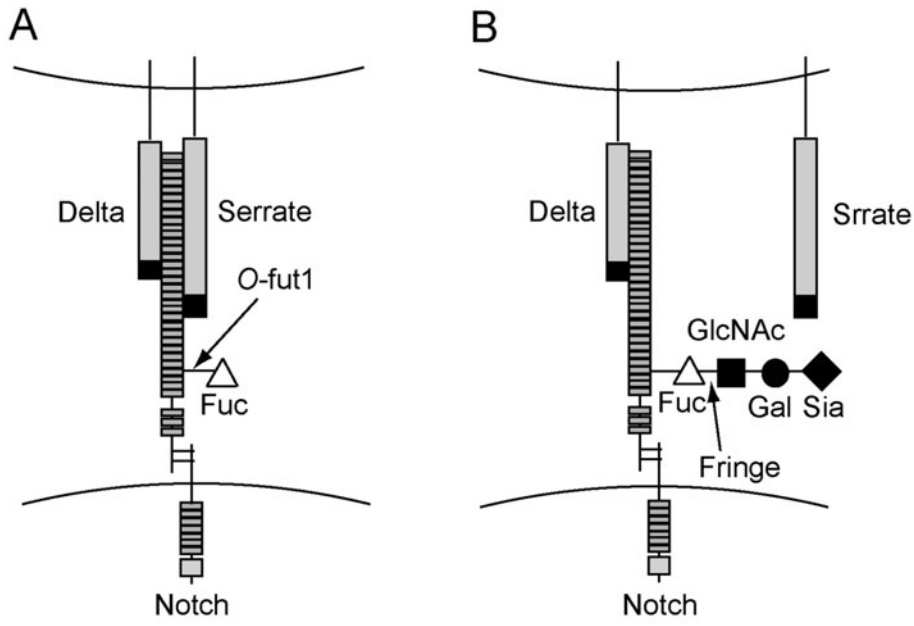


Figure 2. The *O*-fucose glycan modification of Notch regulates the binding specificity between Notch and its ligands. A) *O*-fucosylated Notch by *O*-fut1 binds to Delta and Serrate ligands equally well. B) *O*-fucose is further modified by Fringe and two glycosyltransferases. Notch with the *O*-linked tetrasaccharide modification binds to Delta but not Serrate.

THE MONOSACCHARIDE *O*-FUCOSE MODIFICATION ON THE EGF-LIKE REPEATS OF NOTCH DOES NOT HAVE A MAJOR ROLE IN NOTCH SIGNALING

Genetic analyses revealed that *O*-fut1 orthologs are essential for Notch signaling in all the developmental contexts examined so far in *Drosophila* and mouse.[9,13,17] In contrast, Fringe-dependent Notch signaling occurs in a highly tissue-specific manner.[14,18] Therefore, the monosaccharide *O*-fucose modification of the Notch EGF-like repeats was believed to be essential for the activation of Notch signaling in general.[9] However, it was recently shown that *O*-fut1 has activities that are independent of its enzymatic functions in *Drosophila*.[19,20] Thus, the general requirement for *O*-fut1 in Notch signaling might be owing to *O*-fut1 having essential enzymatic activity-independent functions, rather than to the monosaccharide *O*-fucosylation of Notch EGF-repeats.

In *O-fut1* mutant cells in vivo, Notch accumulates in intracellular compartments that partially overlap with the ER.[19,20] This accumulation is not due to the absence of Notch fucosylation, because it is not observed in flies homozygotic for the *Gmd* mutation, in which *O*-fucosylation is abolished[20] and the mutation of *O-fut1* alsoresults in the accumulation of Notch in the *Gmd* homozygote.[20] These results suggest that this accumulation of Notch in intracellular compartments is induced by the lack of an enzymatic activity-independent function of *O*-fut1.

One of *O*-fut1's enzymatic activity-independent roles is as a chaperon for Notch. The over expression in cultured *Drosophila* cells of fucosyltransferase-dead *O-fut1*, which has a single amino acid substitution mutation in its GDP-fucose binding site, promotes the secretion into the medium of a mutant form of Notch that consists only of its extracellular domain.[19]

Furthermore, the expression of an inactive mutant form of α-glucosidase I, a protein involved in quality control in the ER, rescues the attenuation of Notch signaling activity in mouse *O-fut1* knockout cells.[21] This finding suggests that *O*-fut1 acts as a specific chaperon for Notch in an enzymatic activity-independent fashion. In addition, *O*-fut1 cell-non autonomously promotes the endocytosis of Notch in *Drosophila*, which is also independent of its enzymatic activity.[20] However, the physiological role of this *O*-fut1 function remains to be understood.

These results raise the possibility that the global requirement for *O-fut1* in the activation of Notch signaling can be explained by enzymatic activity-independent functions of *O*-fut1. Thus, the monosaccharide *O*-fucose modification of the Notch EGF-like repeats may not have a specific function, except to provide a base for the GlcNAc modification by Fringe, which tissue-specifically modulates Notch-ligand interactions. This idea is also supported by the observation in *Drosophila* that the neuronal hyperplasia of embryos homozygous for *O-fut1* and lacking its maternal contribution is rescued by the expression of the fucosyltransferase-dead *O-fut1* under the control of its endogenous promoter.[22] However, these phenotypes are accompanied by a recessive lethalmutation and need to be compared with those of *fringe* before a firm conclusion can be drawn.

METABOLIC PATHWAY FOR THE *O*-FUCOSE MODIFICATION OF NOTCH EGF-LIKE REPEATS

The fucose modifications of proteins are classified into two groups: fucosylations of *N*-glycan or *O*-fucosylation.[7,23] These different fucosylations are catalyzed by specific fucosyltransferases, such as FucT[24] and *O*-fut1[7] and require GDP-fucose as a fucose donor. In vertebrates, two independent pathways for synthesizing GDP-fucose are known: the de novo and salvage pathways.[25] In the de novo pathway, GDP-fucose is synthesized from GDP-mannose;[26,27] in the salvage pathway, it is synthesized from fucose.[28] However, based on genome sequencing analysis, homologous genes encoding enzymes for the salvage pathway have not been found in invertebrates.[29] Because the salvage pathway exists in vertebrates, GDP-fucose synthesis is not abolished in animals with mutations in the genes encoding components of the de novo pathway. Thus, knockout mice of *GDP-4-keto-6-deoxymannose3*, *5-epimerase-4-reductase* (also known as *FX*), which encodes an essential enzyme of the de novo pathway, are viable.[30] The oral administration of fucose to *FX* mutant mice suppresses their phenotypes associated with the reduced fucose modifications, because the level of GDP-fucose generated by the salvage pathway is increased by this treatment.[30]

In contrast to vertebrates, *Drosophila* does not have the salvage pathway (Fig. 3).[29] Thus, mutations of genes encoding the enzymes in the de novo pathway result in the absence of GDP-fucose in this organism. *GDP-mannose 4, 6-dehydratase* (*Gmd*) encodes a key enzyme for the de novo synthesis of GDP-fucose.[31,32] The *Gmd* homozygote is lethal at the larval stage, probably because *Gmd* mRNA is maternally provided from

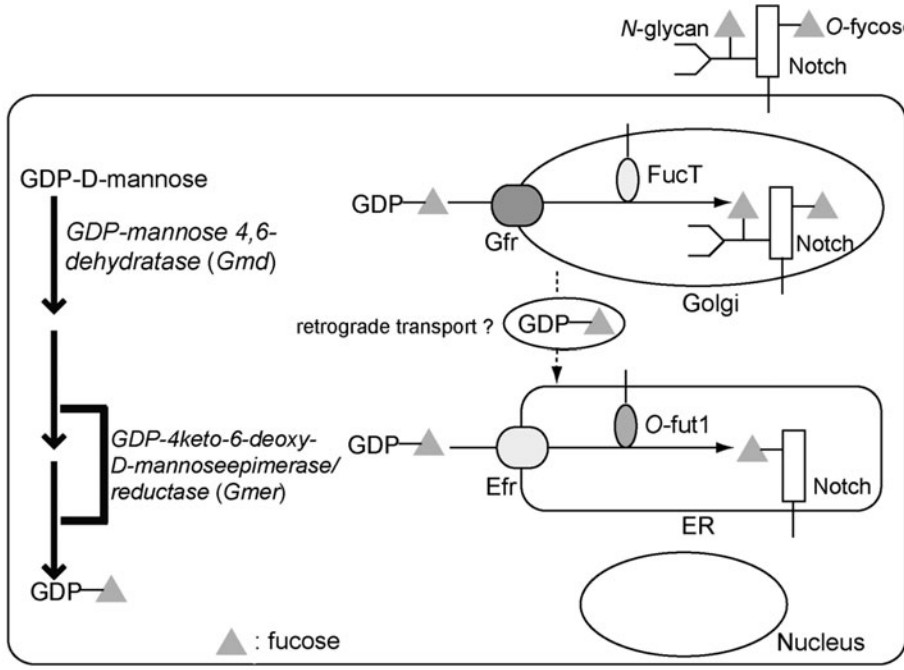


Figure 3. Metabolism and intracellular transport of GDP-fucose, which is required for the O-fucosylation of Notch EGF-like repeats. In *Drosophila*, GDP-fucose is synthesized from GDP-mannose in the cytoplasm *via* the de novo pathway, through two stepwise enzymatic reactions catalyzed by Gmd and GDP-4keto-6-deoxy-D-mannoseepimerase/reductase (Gmer), also called FX. GDP-fucose is transported into the lumens of the ER and Golgi by two GDP-fucose transporters, Efr and Gfr, which are specifically localized to the ER and Golgi, respectively. Efr and Gfr redundantly function for the *O*-fucosylation of Notch EGF-like repeats by *O*-fut1, which occurs in the lumen of the ER. GDP-fucose that is imported into the Golgi lumen by Gfr is retrogradely transported from the Golgi to the ER. Gfr, but not Efr, also play a major role in the fucosylation of *N*-glycans, which occur in the Golgi lumen, by FucT.

the heterozygotic female to the embryos, which is sufficient to support their early development.[19] A biochemical quantification revealed that GDP-fucose is undetectable in *Gmd* homozygote larvae.[20] Fucose modifications of bulk proteins can be detected by Aleuria Aurantia lectin (AAL) in *Drosophila* larval organs, such as wing imaginal discs[33] and AAL staining is largely abolished in the wing imaginal disc of the *Gmd* homozygote.[34]

In addition, the expression of a target gene of Fringe-dependent Notch signaling, *wingless* (wg), is diminished in these organs (Fig. 4B).[20] *wg* encodes a ligand for Wnt signaling and its function is required for the normal development of the wing. In the wing imaginal disc, the ectopic expression of *fringe* induces the ectopic activation of Notch signaling in wild-type *Drosophila.*[33,35] However, this ectopic activation of Notch signaling by *fringe* expression is not observed in *Gmd* homozygotes.[35] Thus, Fringe fails to modulate the activation of Notch signaling in the seflies. These results suggest that GDP-fucose synthesis is essential for the *O*-fucosylation of Notch and the Fringe-dependent activation of Notch signaling.

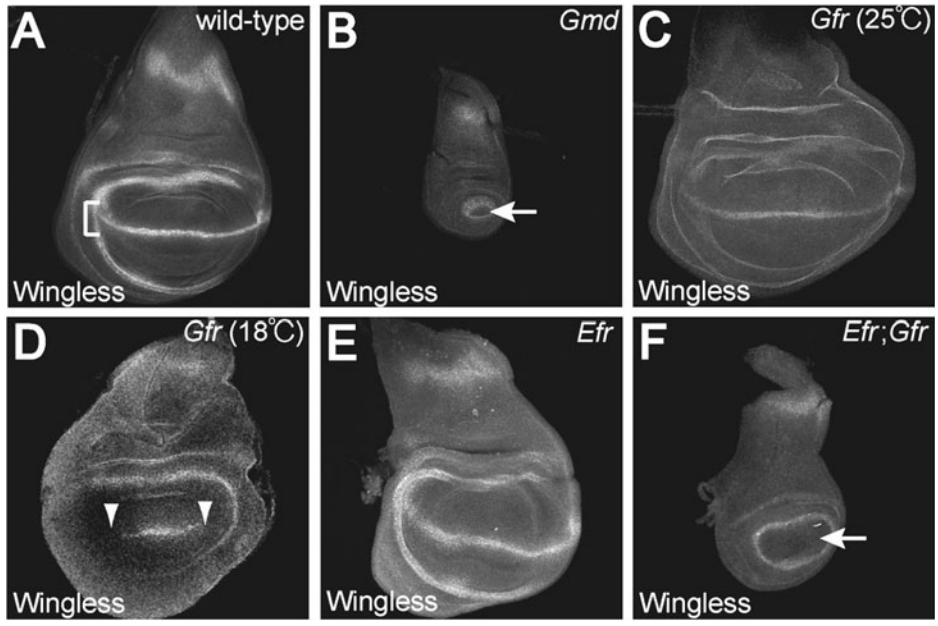


Figure 4. The activation of Fringe-dependent Notch signaling requires GDP-fucose synthesis and its transportation by GDP-fucose transporters, Gfr and Efr. A) In wild-type, the induction of *wingless* (*wg*) gene expression asa stripe acrossthe wing imaginal disc (indicated by white bracket) requires the activation of Fringe-dependent Notch signaling. B) In the wing disc of the *Gmd* homozygote, the expression domain of *wg* is diminished (arrow). (C and D) In the wing disc of the *Gfr* homozygote, the expression domain of *wg* is normal at 25°C (C) but reduced at 18°C (D, arrowheads). These results suggest that Gfr is required for the full activation of Fringe-dependent Notch signaling. E) In the wing disc of the *Efr* homozygote, the expression of *wg* is normal at 18° and 25°C. F) In the wing disc of the *Gfr* and *Efr* double-homozygote, the expression of *wg* is diminished (arrow) at 18° and 25°C. Taken together, these results indicate that *Gfr* and *Efr* function redundantly in the *O*-fucosylation of Notch.

INTRACELLULAR TRANSPORTATION OF GDP-FUCOSE IS CRUCIAL FOR THE *O*-FUCOSYLATION OF NOTCH EGF-LIKE REPEATS

Although GDP-fucose is synthesized in the cytosol, enzymatic fucosylation reactions occur in the lumen of the ER and Golgi. Therefore, GDP-fucose needs to be transported from the cytosol into the lumen of the ER and Golgi (Fig. 3). *GDP-fucose transporter* was identified as a gene whose defect is responsible for the congenital disorder glycosylation Type IIc (CDG IIc), which is alsotermed leukocyte adhesion deficiency Type II (LADII). CDG IIc is characterized by mental and physical retardation, characteristic facial stigmata, severe immunodeficiency and the lack of fucosylated glycans.[36,37] The GDP-fucose transporter is a ten-pass transmembrane protein that is classified as solute carrier family 35C1 (SLC35C1) and localizes specifically to the Golgi membrane.[38] The transport of GDP-fucose through the GDP-fucose transporter is coupled with the export of GMP from the Golgi lumen.[39,40]

The immunodeficiency associated with CDG II cresults from a lack of the carbohydrate epitopes Lewis^X and sialyl-Lewis^X in CDG IIc patients. These two glycans have fucosyl residues and are structural elements of select in ligands, which are required for leukocyte

adhesion and rolling.[41] In contrast, these patients' developmental defects may be owing to the disruption of Notch signaling, becausethe *O*-fucosylation of Notch is required for the Fringe-dependent regulation of Notch signaling, as discussed above.

The *Drosophila* homolog of *SLC35C1* is designated *Golgi GDP-fucose transporter* (*Gfr*), because its gene product is localized to the Golgi.[33] Gfrcan transport GDP-fucose in vitro.[33] The homozygote of the *Gfr*-null mutant is viable and does not show detectable defects at 25°C (optimal growth condition) (Fig. 4C), except for a very mild wing vein phenotype reminiscent of weak *Notch* loss-of-function mutants.[33] However, at 18°C, the *Gfr* homozygote shows a loss of the wing margin, suggesting the attenuation of Notch signaling.[35] Under this condition, the expression domain of the Notch target gene *wg* is reduced in the wing imaginal disc (Fig. 4D) and *fringe* fails to modulate the activation of Notch signaling.[35] These results suggest that the reduction of Notch *O*-fucosylation attenuates Notch signaling in the *Gfr* homozygote at 18°C.[35] Interestingly, however, the fucose modifications of *N*-glycansare severely reduced at both 18° and 25°C in the *Gfr* homozygote, indicating that *Gfr* has a major, but temperature-insensitive, role in their fucosylation.[33] Therefore,the fucosylation of *N*-glycans may not be essential for Notch functioning.

A REDUCTION IN NOTCH SIGNALING ACTIVITY MAY ACCOUNT FOR THE PATHOLOGY OF CDG IIC

Notch signaling is slightly reduced in *Drosophila Gfr* mutants. Thus, Notch signaling may also be attenuated in CDGIIc, in which the human ortholog of *Gfr* (SLC35C1) is genetically disrupted, as mentioned above.[33] This possibility was addressed by knocking down SLC35C1 in mammalian cells. The expression of a short-hairpin RNA corresponding to the SLC35C1 mRNA suppressed the ligand-dependent activation of Notch signaling in C2C12 cells.[33] Thus, a reduction in Notch signaling activity may account for the pathology of CDG IIc that results in developmental abnormalities.

MECHANISMS REQUIRED FOR THE INTRACELLULAR SUPPLY OF GDP-FUCOSE FOR THE *O*-FUCOSYLATION OF THE NOTCH EGF-LIKE REPEATS

In *Drosophila Gfr*-null mutants, the Fringe-dependent activation of Notch signaling is only reduced, not abolished, implying that a low level of *O*-fucosylation is maintained under this condition.[33] Therefore, GDP-fucose must be transported into the ER lumen though an alternative mechanism, suggesting the presence of other GDP-fucose transporter(s) in *Drosophila*. Recently, another GDP-fucose transporter that specifically localizes to the ER was identified and designated ER GDP-fucose transporter (Efr).[35] Efr is a multi functional nucleotide sugar transporter that is involved at least in the biosynthesis of heparan sulfate-glycosaminoglycanchains and the *O*-fucosylation of Notch.[35] A comparison of defects in the fucose modifications of *N*-glycans in *Gfr* and *Efr* mutants revealed that *Gfr* and *Efr* make distinct contributions to these modifications: *Gfr* but not *Efr* is crucial for the fucosylation of *N*-glycans.[35] In addition, *Gfr* and *Efr* function redundantly in the *O*-fucosylation of Notch (Fig. 4C, E and F), even though they have different localizations and nucleotide sugar transportation specificities.[35]

Although *O*-fucosylation occurs in the ER, Gfr and the human GDP-fucose transporter are localized to the Golgi.[33,38,42] Thus, GDP-fucose is probably supplied via retrograde transportation from the Golgi to the ER (Fig. 3). In contrast, Efr probably directly transports GDP-fucose to the ER (Fig. 3). Thus, two pathways for the nucleotide sugar supply, involving two nucleotide sugar transporters with distinct characteristics and distributions, contribute to the *O*-fucosylation of Notch. The symptoms of CDG IIc are less severe than the defects predicted from the phenotype of the *FX* mutant mouse.[30] Therefore, other GDP-fucose transporters that redundantly function with SLC35C1 may be present in mammals, although such transporters still need to be identified.

CONCLUSION

Studies on Notch glycosylation have provided paradigms for the contribution of glycosylation to the functions of transmembrane receptors in general. However, the roles of these sugar modifications are still largely elusive, from a structural biology viewpoint. In the future, a better understanding of the three-dimensional structures of the glycosylated forms of Notch will be needed to help elucidate the molecular bases of the differential binding between Notch and its ligands.

It will also be important to develop new reagents for the artificial manipulation of Notch glycosylations and their functions. These drugscould provide a means for regulating the activity of Notch signaling in patients with diseases associated with aberrant Notch signaling. The development of drugs targeting the roles of these glycans is an important upcoming challenge in this research field.

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CHAPTER 4

NOTCH SIGNALING AND THE GENERATION OF CELL DIVERSITY IN *DROSOPHILA* NEUROBLAST LINEAGES

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Abstract Notch is a membrane bound transcription factor and it plays fundamental roles in many cell-cell interaction events usually involving directly neighboring cells relating an extrinsic signal of a sending cell to the nucleus of the receiving cell to modulate gene expression patterns in this cell. Notch regulates cell fate specification, cell proliferation as well as cell death in the contexts of many organs and cell types. Although the mechanisms of signal transduction from the cell surface to the nucleus are relatively simple, it is not fully understood how such a straightforward pathway can result in tremendously complex outcomes at the cellular level. This chapter discusses some of the known roles of *Notch* during central nervous system development in *Drosophila*. In the CNS, *Notch* is a major player in creating cellular diversity on the level of binary cell fates by possibly activating differential gene expression in sibling cells arising through asymmetric cell division. This chapter also raises some questions related to Notch function during neural cell fate specification which have not yet been satisfactorily addressed in the field. Finding answers to these questions may provide further insights into how cell-cell interactions in the nervous system involving Notch control the generation of cellular diversity.

INTRODUCTION

The brain is the most complex organ on the cellular as well as functional level comprising of about 1.7×10^{11} total cells in human, with roughly an equal composition of neurons and glia and with an estimated number of about 1×10^{15} synaptic connections between the neurons.[1] One of the most intriguing but still largely unresolved problems in neurobiology is how this cellular complexity is generated during development through mechanisms such as

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cell proliferation, cell-cell interactions, cell specification, cell differentiation and cell death. *Drosophila* has been proven for decades to be an ideal model system to resolve problems on the level of single identified cells in the nervous system. It has been demonstrated in the fly that Notch plays a fundamental role at two levels of neurogenesis: (1) Notch is critical during the phase when neuroblasts are singled-out from the neuroectoderm by a process called lateral inhibition of cells in equivalence groups and (2) Notch functions during later stages of nervous system development in conjunction with asymmetric cell division to regulate differential binary cell fate specification of sibling cells.

NOTCH IS REQUIRED FOR LATERAL INHIBITION DURING THE PHASE OF NEURAL STEM CELL GENERATION

In the fruitfly, the nervous system derives largely from neural progenitors which are called neuroblasts (NBs). Neuroblasts are the equivalent of neural stem cells of higher organisms as they are able to self-renew and give rise to differentiated progeny, two major hallmarks of stem cells. During embryonic development of the ventral nervous system, approximately 30 NBs are generated per thoracic/abdominal hemisegment and the Notch signaling pathway plays a fundamental role in selecting individual neuroectodermal cells to become a neuroblast by a mechanism termed lateral inhibition. In this context, Notch signaling is required to repress most of the cells of a group of equally potent cells (equivalence group) to become a neuroblast and to adopt an ectodermal cell fate instead (Fig. 1A). Thus,

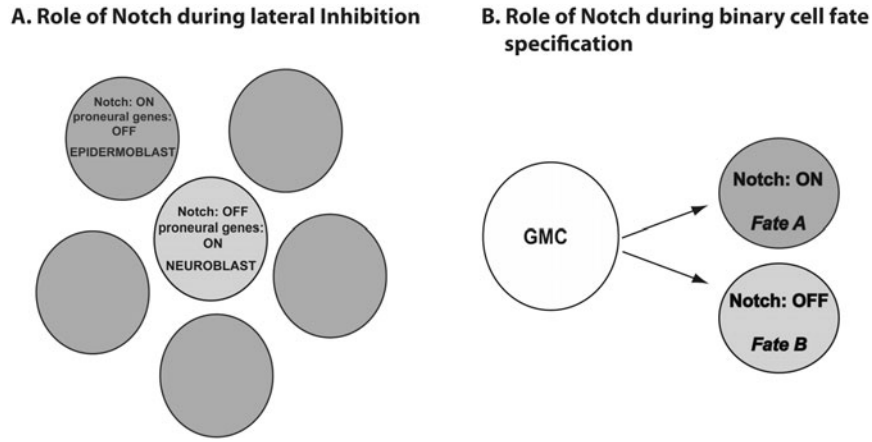


Figure 1. Notch plays two major roles during nervous system development in *Drosophila*. A) Role of Notch during lateral inhibition in the neuroectoderm. B) Role of Notch during binary cell fate specification in postmitotic cells. A) Notch signaling represses the neural fate in epidermoblasts. As a consequence, cells with active Notch signaling develop an epidermal fate. On the molecular level, this is achieved through the repression of the proneural genes. Proneural genes are activated in the neuroectodermal cells lacking Notch signaling and as a result such cells become neuroblasts. Neuroblasts are the neural stem cell equivalent of the fly and produce the entire nervous system in a lineage dependent manner. B) The terminal division of individual GMCs leads to two distinct cell identities, here termed fate A and fate B. One of these fates strictly requires Notch signaling for its correct specification whereas the other sibling only develops correctly in the absence of functional Notch signaling. The concept of Notch as a crucial discriminator of binary sibling cell fates seems to apply during embryonic as well as postembryonic neurogenesis in *Drosophila*.

Notch acts as a classical repressor of neurogenesis during the phase in which neuroblasts are selected from the pool of neuroectodermal cells. This is reflected by the fact that *Notch* mutants display a hypertrophic nervous system in which most of the neuroectodermal cells convert to neuroblasts at the expense of epidermoblasts.[2-4]

Notch generally acts as a transmembrane transcription factor. In response to ligand binding its intracellular domain gets cleaved off and then translocates to the nucleus.[5,6] In conjunction with Suppressor of Hairless (Su-(H)), Notch binds DNA and induces expression of down-stream target genes such as the Enhancer-of-Split (E-(Spl)) complex[7] resulting in fundamental changes of gene expression in the signal receiving cells.

ASYMMETRIC CELL DIVISION AS A MECHANISM TO GENERATE CELL DIVERSITY IN NEUROBLAST LINEAGES

A NB undergoing cell division normally regenerates a new neuroblasts and at the same time gives rise to a secondary precursor cell called ganglion mother cell (GMC). Each GMC typically divides only once to generate two postmitotic sibling cells which differentiate into neurons and/or glial cells, or undergo programmed cell death. Cell lineage analysis has demonstrated that each NB generates a stereotypic set of unique and mostly identifiable daughter cells[8-11] referred to as the neuroblast lineage. Therefore, mechanisms of cell fate specification in the fruitfly are strictly linked to the NB lineage context and the relatively simple neural stem cell lineages of the fly are ideal model systems to study the biology of neural stem cells. During each division NBs as well as GMCs divide in a polarized, asymmetric fashion producing progeny with binary identities[12] such as a new neuroblast and a GMC in the case of NB divisions or two sibling neurons and/or glial cells in the case of GMC divisions. As such, asymmetric cell division provides a key mechanism for the generation of cell diversity.

Intrinsic as well as extrinsic cues play crucial roles in the specification of distinct sibling cell fate identities. The intrinsic mechanisms are tightly linked to asymmetric cell division and involve the coordinated control of spindle orientation ultimately leading to highly polarized cells during cell division. As a result of this polarity, cellular determinants such as mRNAs and proteins are asymmetrically distributed into specific compartments of the dividing cell prior to cytokinesis. This leads to unequal distribution of determinants preferably only into one of the two daughter cells rendering the two siblings intrinsically different. Cell polarity is mediated by an apically localized protein complex which includes Insc;[13,14] the Par proteins: Bazooka (Baz),[15,16] DaPKC[16] and DmPar6,[17] a protein cassette involved in heterotrimeric G protein signaling: GαI,[18] Partner of Inscuteable (Pins)[19-21] and Locomotion defects.[22] Insc has been described as a major organizer of the apical complex controlling apical-basal spindle orientation, basal localization of protein determinants such as Prospero and Numb as well as asymmetric size of the daughter cells by coupling cell polarity to spindle positioning pathways thereby controlling downstream sibling cell fate specification via the extrinsic mechanisms.[23,24]

A part of the extrinsic mechanisms is the activation of the Notch signaling pathway which critically depends on the correct asymmetric distribution of Numb into only one of the two siblings. Numb physically interacts with Notch and negatively regulates Notch signaling in only the sibling which inherits the Numb protein. Thus, the Notch receptor provides a link between intrinsic and extrinsic mechanisms during sibling

cell fate specification. As a consequence of asymmetric cell division and activation of Notch signaling in only one sibling, the fate of one sibling strictly depends on functional Notch signaling (the *Notch*-dependent sibling; N^{dep}) whereas the other sibling (the *Notch*-independent sibling; N^{indep}) adopts a default fate as it differentiates correctly only if Notch signaling is not present. The event of permitting or inhibiting Notch signaling in the respective postmitotic sibling through the asymmetric segregation of the intrinsic cell fate determinant Numb seems to be crucial and sufficient for correct sibling cell fate specification, therefore assigning an important role to Notch signaling in the process of binary cell fate specification. In this context Notch can be regarded as an effector of asymmetric cell division leading to the realization of differential gene expression patterns in the two sibling cells. This will be discussed in more detail in the next paragraph.

FUNCTION OF NOTCH IN BINARY CELL FATE SPECIFICATION IN THE *DROSOPHILA* NERVOUS SYSTEM

Numb and Notch have been shown to affect binary sibling cell fate choices in some pairs of sibling cells in the embryonic nervous system of *Drosophila*. The first demonstration that Numb via modulating Notch signaling affects sibling cell fate specification was shown in a simple neuroblast lineage, the MP2 lineage which gives rise to only two sibling daughter cells, the vMP2 and the dMP2. Loss of *Notch* resulted in sibling cell fate transformation of vMP2 into its sibling dMP2.[25] Conversely, in *numb* mutants the opposite transformation was observed suggesting that for the specification of the vMP2 functional Notch signaling is required whereas Notch is redundant for the specification of the dMP2 fate.[25] A similar model for Notch, Numb and Mastermind, another component of the Notch signaling pathways was shown to be crucial for resolving sibling cell fate choices for two pairs of siblings, the aCC/pCC neurons derived from the first GMC of NB1-1 and the RP2/RP2sib which derive from the first GMC of another neuroblast, NB-4-2.[26] Similar results were presented by Skeath et al (1998) including additional sibling cell fate pairs from two additional neuroblasts, the U-neurons and their siblings from NB7-1 and the eve-lateral cells (EL) from NB3-3.[27]

Furthermore, it was shown that a subset of glial cells, the superineurial glia (SPG) in the embryonic nervous system was a result of asymmetric divisions of the respective GMCs, including three GMCs from NB1-1. In this context, Notch signaling was required to generate the SPG glia. Absence of *Notch* led to an almost complete loss of SPGs[28] as well as to a duplication of the neuronal siblings. It was also shown that in SPGs Notch acts genetically upstream of *glial cells missing* (*gcm)*, a master regulator of glial cell fate specification. However, most of the other glia cells which belong to non-SPG subtypes did not require Notch function. This study in summary raised the possibility that Notch is an effector of asymmetric cell division to positively control *gcm* expression in specifying a subset of glial cells, the SPG glia. However, in the peripheral nervous system (PNS) of the adult fly which also involves asymmetric cell division in sensory organ precursors (SOP) and binary sibling cell fate resolution of daughter cells within the SOP lineage, Notch plays an opposite role. In this context, Notch works as a repressor of the glial fate as lack of Notch led to *gcm* expression on the contrary to Notch activating *gcm* expression in the ventral nerve cord.[29] As such, it seems that depending on the cell context Notch may have either an inductive or a

repressive role in terms of glial cell fate specification. The notion of context specificity of Notch function in terms of glial fate specification is further supported by observations that in the PNS there are two distinct SOP lineages in which glial cell specification requires Notch signaling for activating *gcm* expression.[30] It was also demonstrated that Notch activity was strictly restricted to the glial cells by monitoring translocation and transcriptional activity of the Notch intracellular domain. Umesono et al also reported that Nubbin/PDM1 could provide the context specific mechanism by which Notch acts as a promoter of gliogenesis. As such, at least in some SOP lineages the requirement for Notch to induce glial differentiation is similar to the SPG in the embryonic ventral nerve cord as described by Udolph et al.[28] In summary, depending on the cellular context, Notch can either promote or inhibit gliogenesis but the general concept that one of the binary siblings requires Notch while the other one doesn't still applies.

In the effort to analyze Notch requirement during the entire embryonic nervous system development in a NB lineage context, the requirement for *insc,* a master regulator of cell polarity and asymmetric cell division and *Notch* was tested in NB cell lineages. Such NB lineages were derived from cells in which either *Notch* or *insc* was absent. The resulting mutant lineages of were analyzed in great detail to understand the role of *insc* and *Notch* during cell fate specification[31] in a NB lineage context. It was demonstrated firstly that cell fate changes in *insc* mutant NB lineages were far less pronounced as *insc* was only strictly required for cell fate specification for cells that arose from early divisions within these NB lineages. However, *insc* was not required for cell fates arising later in the same lineages. In contrast, *Notch* mutant NB lineages showed cell fate changes throughout the entire lineages also in late born cells in NB lineages showing that Notch was still strictly required for sibling cell fate specification late in NB lineages when Insc was not required anymore. This implies that asymmetric cell division mediated by Insc might be redundant to other components of the apical complex of proteins such as Bazooka or Pins. A scenario like this has been described for the MP2 siblings where *insc* plays a redundant role and asymmetric cell division in these cells depends on Bazooka[32] as an organizer of polarity rather than Insc. Alternatively, later born cells may be less dependent on intrinsic asymmetric cell divisions as mechanisms of cell-cell interactions via Notch may predominate in late phases of lineage development. However, it is currently unknown how sibling cells control the binary mode of activating or repressing Notch signal transduction in the two daughters during later lineage development.

In addition, the lineage data clearly revealed that Notch signaling is not required for other aspects of NB development such as NB specification and NB proliferation. All the lineages developed rather normally in terms of numbers of cells and cell types produced in these lineages. These findings demonstrated that Notch does not control NB lineage progression per se as the mechanisms controlling NB specificity, NB proliferation, mechanisms specifying cell fates of GMCs and the general process of maturation of postmitotic cells were generally unaffected.[31] Similar conclusions have been reached for Notch function in larval NBs in which Notch did not affect proliferation, the maturation of cells as well as stemness of the postembryonic neuroblasts.[33] Lastly, Notch signaling in sequential pairs of postmitotic siblings from the same neuroblast during embryo neurogenesis can result in diverse cell specification raising the question about the underlying mechanisms of sequential cell fate specification and the role of Notch in this process.

NOTCH FUNCTION IN CELL FATE SPECIFICATION IN THE EMBRYONIC NB7-3 LINEAGE

Further insight into the role of Notch signaling in binary cell fate specification came from studying a simple neuroblast lineage, that of NB7-3. NB7-3 initially gives rise to a total of 6 cells consisting of 4 neurons (two serotonergic neurons, one corazoninergic neuron and one motorneuron) plus two cells undergoing apoptosis.[34,35] The two serotonergic neurons are derived from GMC1 and GMC2 whereas the corazoninergic neuron is produced by GMC3. The three interneurons are derived from the N^{indep} siblings of the three GMCs whereas the motorneuron and the two apoptotic cells are N^{dep} cells.[35] This suggests that Notch suppresses the serotonergic as well as corazoninergic differentiation programme but supports the specification of the motorneuron as well as promotes cell death in two cells. At least in the context of NB7-3, *numb* seems to have a minor role in regulating Notch signaling in the respective postmitotic siblings as in *numb* mutants the expected binary cell fate conversions were not observed suggesting the existence of redundant numb-like factors facilitating the inhibition of Notch signaling in these cells. Further to that, it has been shown that *Notch* and *eagle* regulate the expression of *Zfh-1*, a gene which is found to be expressed in the N^{dep} cells of the NB7-3 lineage.[36] As such, Zfh-1 could be a potential Notch target gene (NTG).

NOTCH FUNCTION DURING LARVAL NEUROGENESIS

Notch function is also required during the phase of larval neurogenesis. Larval neurogenesis is initiated when quiescent embryonic neuroblasts resume proliferation and start to produce a set of daughter cells that as a group is referred to as secondary cells. In larval neurogenesis Notch is also important for binary cell fate specification of siblings in many if not all secondary postmitotic sibling pairs of neurons.[37] It was reported that similarly to the embryonic nervous system, Notch plays role in binary sibling cell fate choice whereby cells with active Notch signaling assumed one particular cell fate whereas cells without Notch signaling assumed another fate.[37]As such, throughout larval neurogenesis, Notch generates two hemilineages within a NB lineage as Notch signaling clearly separates each NB lineage into two set of relatively identical sublineages (Fig. 2). This concept of hemilineages implies that generally Notch signaling divides the entire nervous system of *Drosophila* into two subsets of cells: one set requiring Notch signaling for correct specification and the other set does not require Notch. Interestingly, the uniform, monocytic lineages which are commonly produced during larval neurogenesis were found to be a consequence of one of the hemilineages undergoing apoptosis[37] although apoptosis was not strictly related to either presence or absence of Notch signaling in different NB lineages. Supporting earlier findings in the embryonic NB7-3 lineage,[35] it was also shown that *numb* was not a critical component during larval neurogenesis. Therefore, yet to be identified Notch repressors in the context of asymmetric cell division must exist during embryonic as well as larval nervous system development. This set of data also underlined that cell death has to be regarded as a binary cell fate choice during asymmetric cell division. Such notion is also supported by a study by Lundell et al describing a role for Notch in apoptosis in the embryonic NB7-3 lineage.[35]

In summary, Notch signaling is a crucial mediator of binary cell fate specification in cells resulting from asymmetric cell divisions. Notch can have opposing functions as it may facilitate as well as suppress glial cell fate. How these opposing functions are controlled on

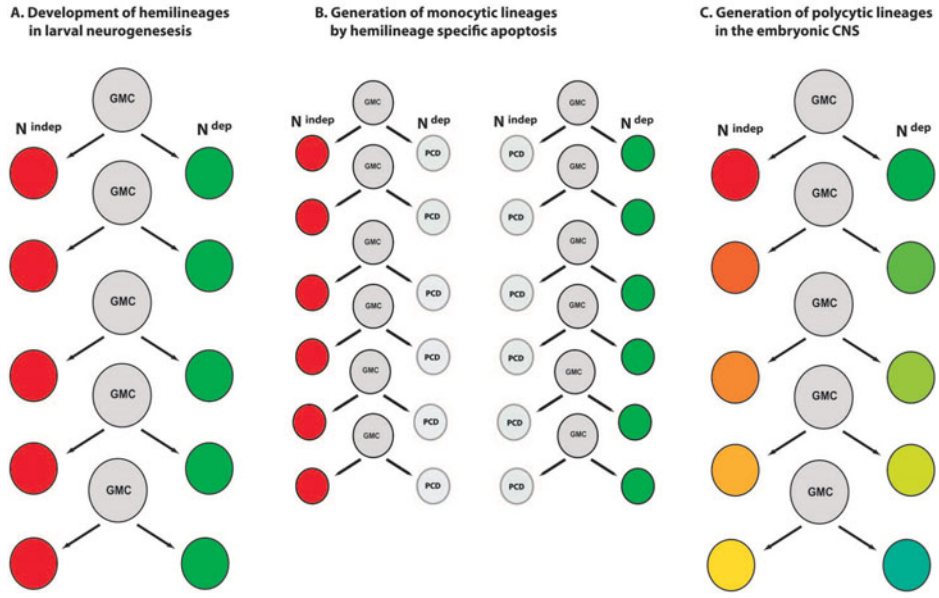


Figure 2. The development of cell diversity during *Drosophila* neurogenesis. A) During larval neurogenesis, Notch signaling divides the NB lineage into 2 hemilineages each consisting of the subset of either the N^{dep} or N^{indep} siblings. The hemilineage concept might help to provide better understanding on how genes can control the construction of functional units in the brain.[37] B) Some larval neuroblasts generate monocytic lineages which only contain a single cell type. Hemilineage specific apoptosis might lead to the generation of such monocytic lineages whereby one hemilineage undergoes programmed cell death. In larval neurogenesis, a correlation between programmed cell death (PCD) and Notch signaling is not evident. C) In the embryo, NBs lineages generally form polycytic lineages consisting of many different and distinct cell types indicated by different shades of color. Such lineages can be considered as consisting of 2 polycytic hemilineages in which each hemilineage generates different cells fates at each level in the N^{dep} as well as in the N^{indep} hemilineage. The mechanisms of how Notch signaling produces distinct cell fates in embryonic hemilineages are currently only poorly understood. N^{dep}, Notch dependent; N^{indep}, Notch independent; PCD, programmed cell death.

the level of single cells is currently unknown. Notch signaling divides neuroblast lineages into two hemilineages and conceptually this might help to understand how genes control units of connectivity in the nervous system.[37] At least in the embryonic nervous system, the Notch dependent hemilineages can contain many diverse cell types and the question arises how such differential cell fate specification in the Notch dependent hemilineages is achieved.

Is There Contextual Control of Notch Activity?

As described above, Notch signaling creates or facilitates distinct differentiation outcomes in different postmitotic cells. Under the assumption that differential cell fate requires differential gene expression, it can be postulated that Notch signaling has to function in a contextual manner and final outcome in terms of cell differentiation as a result of Notch signaling depends on other factors. The nature of such factors, however, remains largely unknown.

However, it has been demonstrated that genes can be expressed at different levels in the neuroblast lineage with a strong temporal dependency. It was shown that genes such

as the POU genes (also termed pdm-1 and 2) as well as castor (*cas*) and hunchback (hb) established layered compartments in the CNS during development suggesting temporal control of their gene expression in NB lineages depending on the birth order of the cells that arise from these NB lineages. It was suggested that the early cells in neuroblast lineages express *hb*, intermediate cells express *pdm-1* and late lineage components express *cas*.[38] Further insight into the importance of such temporal expression of genes came from studying two of the best understood neuroblast lineages, NB7-3 and NB7-1, for which most of the cell types within these two lineages are known on a single cell level. For example, the NB7-1 produces the five Even-skipped (eve) positive U-neurons (U1-5) and their respective Eve-negative siblings (Usib1-5) from the first five Eve-positive GMCs. It was shown in a landmark study[39] that NB7-1 (as well as NB7-3) sequentially expressed transcription factors such as Hb, Krüppel (Kr), Pdm and Cas during their development and sequential NBs within the same lineage expressed Hb/Kr, Kr, Pdm and then Pdm/Cas (Fig. 3). In analogy to sequential gene expression in the neuroblasts, the Hb/Kr positive

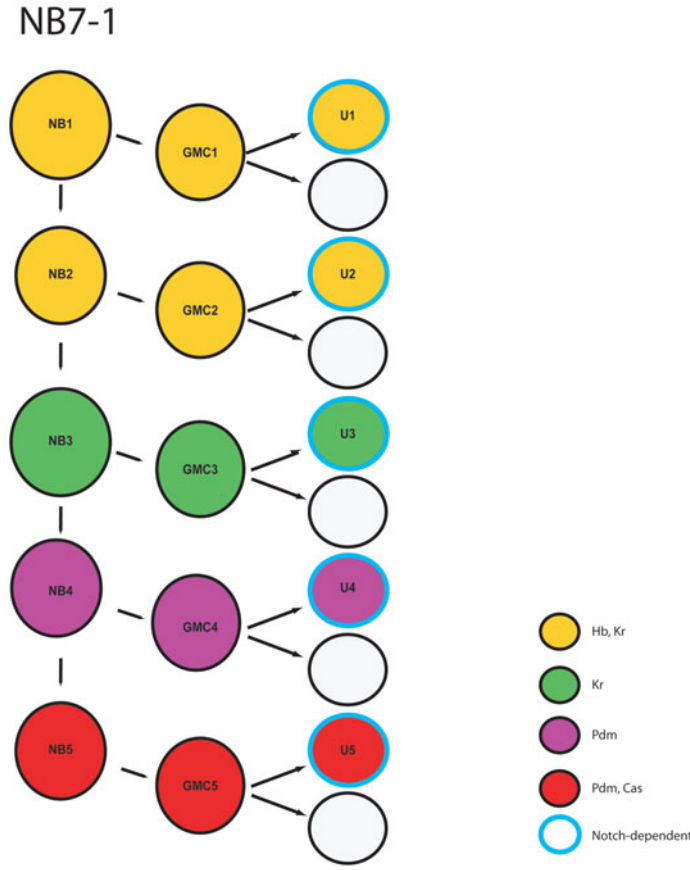


Figure 3. Temporal expression of genes in NB7-1 lineage. Temporally expressed genes such as *Hb*, *Kr* and *Pdm* in NB lineages could provide a contextual framework for Notch signaling contributing to distinct fate specification of N^{dep} cells as exemplified by the specification of the different U-motorneurons (U1-5) generated from the first five GMCs of NB7-1.

NBs produced two Hb/Kr positive GMCs which gave rise to the Hb/Kr positive U1 and U2 neurons, the Kr positive NB generated a Kr positive GMC which produced the Kr positive U3 neuron. GMC4 was born form the Pdm positive NB and gave rise to the Pdm positive U4 neuron whereas the and GMC5 was born from Pdm/Cas positive NB and produced the Pdm/Cas positive U5 neuron.

Thus, differential gene expression in the sequential neuroblasts, GMCs and in postmitotic neurons of the same lineage, specify a temporal identity of cells born at different time points.[39] As a consequence, all the N^{dep} U-neurons (as well as their respective GMCs) are exposed to different contexts of these sequentially expressed genes. It was indeed shown that the level of e.g., Hb expression at different stages of neuroblast lineage progression was instrumental for the differentiation of early versus late cell fates in the NB7-1 lineage.[40] The differential expression of genes providing temporal identity to cells born within the lineage such as Hb, Kr, Pdm and Cas is consistent with the notion that these genes also could provide contextual information for Notch signaling in the respective cells. A possible mechanism for the regulation of the temporal expression of Hb has been suggested by Mettler et al (2006).[41] In this context, a mitosis dependent control of Seven-up translation repressed *hb* expression in NBs which already ran through some rounds of divisions, thus directly linking cell division to the expression of temporal markers in NB lineages.

Possibly another known gene, *klumpfuss* (*klu*), has to be seen in context of controlling temporal NB identity as well. Based on its presence or absence in sequential GMCs of NB4-2, respective postmitotic cells assume differential cell fates.[42] Therefore, Klu in addition to Hb, Kr, Pdm and Cas might contribute to the contextual information in which Notch signaling could generate binary outcomes in sibling cells and differential outcomes in horizontal lineage progression within individual NB lineages. Further studies, however, will be needed to demonstrate the contextual requirement for these genes in the Notch signaling pathway.

DOWN-STREAM NOTCH TARGETS/EFFECTORS

One of the major unsolved problems in explaining the different functional outcomes of Notch signaling during cell fate specification in the nervous system is the lack of potential direct or indirect Notch target genes (NTG). The canonical Notch signaling pathway works through Su-(H) and regulation of the E-(Spl) complex.[7] However, it has been shown that there are Notch-independent functions of Su(H)[43] as well as Su(H) independent Notch signaling.[36] The knowledge about downstream mechanisms of Notch signaling such as potential NTGs as well as regulatory networks in which such genes might be operating are very limited and are currently poorly understood with the list of potential NTGs being surprisingly short to date. Therefore, identifying NTGs and unraveling their mechanisms on how they affect cell fate specification would greatly help in understanding how Notch signaling controls the generation of cell diversity during nervous system development.

Recently, *hey* has been identified as a downstream target of Notch signaling. *hey* is activated in many, if not all Notch responsive cells during embryonic and larval neurogenesis.[44] As expected, in *Notch* mutants *hey* was greatly down-regulated, in *numb* mutants *hey* was observed in more cells and *hey* mutants displayed sibling cell fate choice phenotypes similar to *Notch* mutants. Furthermore, *hey* overexpression was sufficient to affect binary cell fate changes in the predicted direction. This set of data strongly supports the hypothesis that *hey* is a NTG. *hey* belongs to the family of bHLH-O type of transcription factors of which there are 13 know members in the *Drosophila* genome including the seven *E(Spl)* genes.

Interestingly, with the current studies by Monastiroti et al.[44] as well as Krejci et al[45] who performed a genome wide study of Notch targets genes, 9 out of these 13 bHLH-O genes now have been demonstrated to play a role in Notch signaling.

In addition, *Zfh-1* has been identified as a potential NTG in the NB7-3 lineage[36] and *gcm* might be a NTG in the central as well as peripheral nervous system in some glial subtypes [28,30]. However, beyond these few NTGs not much is known on how Notch signaling might regulate cell fate decisions in the nervous system. It is critical to identify additional NTGs in order to gain better understanding on cell-specific mechanism of Notch signaling during binary cell fate specification. The identification of NTGs may not be an easy task which is mainly due to the probable context specificity of such genes on the level of single cells. Therefore, the comprehensive identification of NTGs in binary cell fate specification could turn out to be challenging as conventional tools of genome wide analysis and high-through-put assays generally lack sufficient resolution to study mechanisms on a single cell level. To proceed, it is imperative that appropriate markers and assays are developed in order to address these questions probably on the level of single cells or at least at the level of group of cells like glia or subtypes of neurons.

Is Notch Instructive or Permissive?

There is an ongoing and currently unresolved debate in the field whether Notch works instructively by directly influencing cell fate specification or whether Notch sets a permissive environment in which other genes can exert their specific functions. During binary cell fate decision it is also not clear which role Notch might play. Direct downstream targets of Notch have been identified such as *hey* and other members of the bHLH-O family of transcription factors. Most of these known targets share Su(H) binding sites in their promoters[44] and this might suggest that these genes are directly regulated by Notch, indicating an instructive role for Notch. The mechanisms how any downstream target is activated and whether Notch directly and actively drives the transcription of target genes remains to be explored. For example, it could well be that Notch/Su-(H) works in a supportive manner allowing other and yet unknown transcription factors to bind to Notch/Su(H) occupied promoters to initiate gene transcription. It is interesting to note that Notch has been shown to be able to recruit cofactors such as histone acetyl transferases[46] or to genetically interact with chromatin-remodeling enzymes such as Brahma.[47] The emerging concept here is that Notch signaling requires chromatin remodeling suggesting that epigenetic changes are either required for or might be a consequence of Notch signaling. Also the context specificity of Notch signaling might suggest that Notch signaling acts in conjunction with other transcription factors and signaling pathways to generate specific cells types. The analysis of the role of Notch signaling in the specification of binary cell fates during NB lineage progression may reveal major insight in the mechanisms how Notch drives the generation of the cellular complexity of the nervous system. Such insights will also impact on the mechanisms of generating cell diversity in other organ systems as well as providing some answers to the question whether Notch works instructively or permissively.

CONCLUSION

Although much is known about cell fate specification on the level of individual cells in *Drosophila* particularly in the embryonic nervous system (summarized in Figs. 4 and 5)

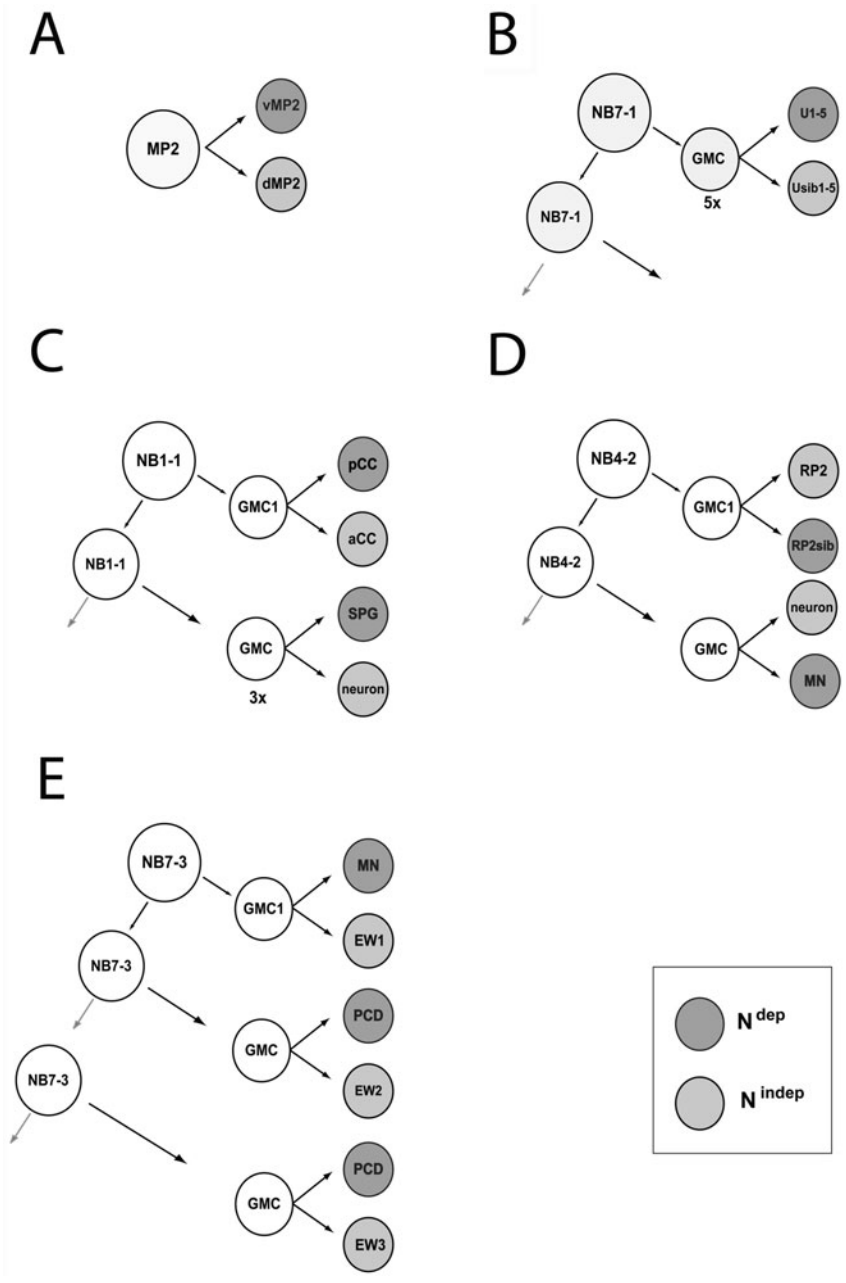
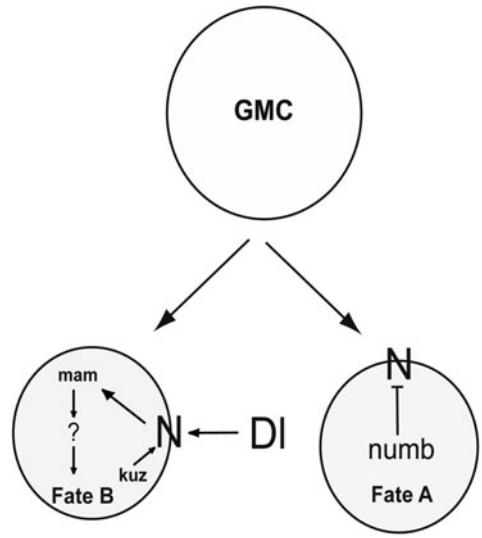


Figure 4. Known cell fates in 5 model neuroblast lineages depicting the N^{dep} as well as the N^{indep} cell types currently known in these lineages. Shown are five NB lineages, A) MP2, B) NB7-1, C) NB1-1, D) NB4-2 and E) NB7-3. Notch dependent cell fates are depicted in green whereas Notch independent cell types are in grey. MN, motorneuron; EW1, serotonergic neuron derived from GMC1; EW2, serotonergic neuron derived from GMC2; and EW3, corazoninergic neuron derived from GMC3; N^{dep}, Notch dependent; N^{indep}, Notch independent; PCD, programmed cell death. (Figure is a modified version taken from Udolph et al, 2009).[31]



NBs:	SibB:	SibA:
MP2	dMP2	vMP2
NB1-1 (GMC1)	aCC	pCC
NB1-1 (3x GMCs)	3x SPG1-1 sibs	3x SPG1-1
NB4-2 (GMC1)	RP2	RP2 sib
NB4-2 (~2 GMCs)	~ 2 MN4-2 sibs	~2 MN4-2
NB7-1 GMC1-5	U1-U5 neurons	U1-U5 sibs
NB7-3 GMC1	EW1 (serotonergic)	GW (motoneuron)
NB7-3 GMC2	EW2 (serotonergic)	PCD
NB7-3 GMC3	EW3 (coraznoninergic)	PCD
some lNBs	?	motorneurons

Figure 5. Summary of the known binary cell fate specification during embryonic nervous system development in *Drosophila*. Ndep and Nindep cell fates are summarized based on the parent neuroblast and the GMCs. It is apparent that activating Notch signaling in one sibling cell and deactivating it in the other is a major driver of the generation of cellular diversity during *Drosophila* embryonic and possibly larval neurogenesis.

still much needs to be learned about the role of Notch signaling down-stream of Notch. *Drosophila* with its advanced molecular and genetic tools will be an ideal system to unravel the mechanisms of how Notch might affect cell fate specification possibly in a contextual manner. It is fair to predict that much will be learned from *Drosophila* about Notch function and how Notch affects transcriptional changes and shapes genetic networks in the fly to generate neural diversity. Given the high similarity and conservation between flies and vertebrates, the potential yield of knowledge about mechanisms of Notch function in generating cell diversity in the nervous system of the fly will hopefully provide further insights into mechanisms contributing to the vast complexity of the vertebrate nervous systems including that of humans.

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CHAPTER 5

NEPRO:
A Novel Notch Effector for Maintenance of Neural Progenitor Cells in the Neocortex

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Abstract: The Notch pathway is essential for maintaining neural progenitor cells (NPCs) in the developing brain. Activation of the pathway is sufficient to maintain NPCs, whereas loss-of-function mutations in the critical components of the pathway cause precocious neuronal differentiation and NPC depletion. Hairy and Enhancer of split (Hes)-type transcription factors have long been thought to be the only Notch effectors for the maintenance of NPCs. Recently, a novel nuclear protein, Nepro, has been identified as another critical effector of Notch. The Notch pathway is bifurcated into Nepro and Hes-type proteins in the early development of the neocortex. The combination of Nepro and Hes-type proteins is necessary and sufficient for maintaining NPCs downstream of Notch.

INTRODUCTION

The neocortex, which is involved in higher cognitive functions in mammals, is composed of six layers. Excitatory projection neurons of the layers are generated from neural progenitor cells (NPCs) residing in the ventricular zone (VZ) of the dorsal forebrain during development (Fig. 1) (for a review see ref. 1). At the beginning of neocortex development, neuroepithelial cells divide symmetrically to increase their numbers (Fig. 1A). At around embryonic (E) 9.5, they convert to radial glial cells (RGCs), which function as NPCs at early stages.[2] Early NPCs divide asymmetrically to give rise to NPCs and neurons or basal progenitors (BPs) (Fig. 1B). Similarly, late NPCs generate NPCs and BPs (Fig. 1C). Neurons migrate into the cortical plate (CP)

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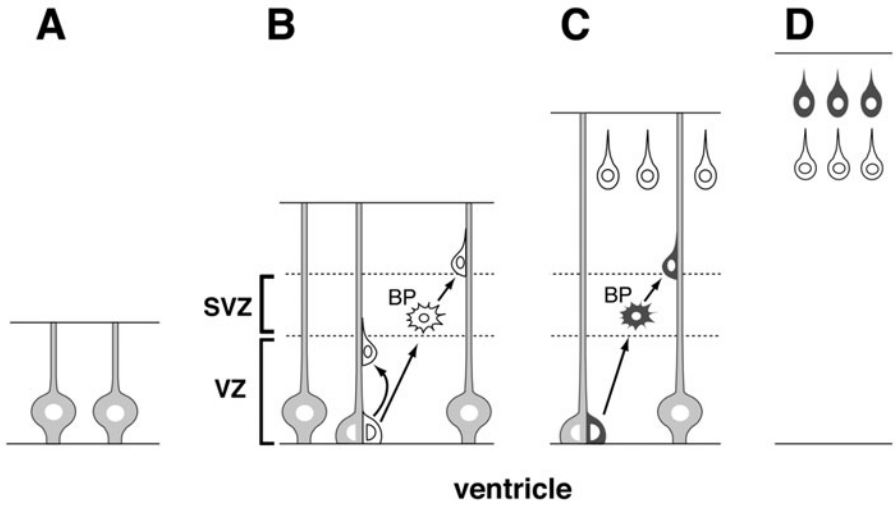


Figure 1. Development of the neocortex. Formation of low layers (IV to VI) and upper layers (II and III) of the mouse neocortex is illustrated schematically. Neuroepithelial cells initially divide symmetrically (A) and subsequently give rise to RGCs at ~E9.5. B) Most RGCs divide asymmetrically to generate RGCs and neurons or BPs between ~E9.5 and ~E14.5. The neurons migrate along RGCs and occupy low layers of the CP. BPs divide symmetrically to form neurons in the SVZ and the neurons migrate as above. C) From ~E14.5 to around the end of embryonic development, asymmetric division of RGCs generates RGCs and BPs. The later BPs undergo symmetric division into neurons that pass through the low layers and accumulate in upper layers. D) The layers of the neocortex are formed in an inside-out fashion in which later-born neurons are situated in more superficial layers.

using radial glial fibers as scaffolds. BPs migrate to the subventricular zone (SVZ) and divide symmetrically to form neurons. Early-born neurons occupy low layers of the CP and later-born neurons migrate into more superficial layers so that layers are sequentially formed from bottom (layer VI) to top (layer II) (Fig. 1D) (for a review see ref. 3).

NPCs change their potential according to embryonic development. The initial NPCs are competent to generate neurons of layers VI to II and NPCs gradually lose the competence to generate neurons of lower layers.[4-6]

At later stages (around the end of embryonic development), RGCs stop generating BPs and instead produce astrocytes, oligodendrocytes, ependymal cells and adult neural stem cells.[2] Therefore, the maintenance of NPCs is essential for the formation and function of the neocortex.

THE NOTCH PATHWAY IS ESSENTIAL FOR MAINTENANCE OF NPCs

Notch signaling plays a pivotal role in the maintenance of NPCs (for a review see ref. 7). Transfection of the constitutive active form of *Notch* (*caNotch*) inhibits neuronal differentiation and maintains NPCs.[6,8] NPCs resume generating neurons after switching off the transfected *caNotch*, indicating that the state of NPCs is not irreversibly fixed by *caNotch*.[6] The neurons generated from the NPCs in which *caNotch* is switched

off show the same phenotypes in terms of molecular markers and layer positions as those generated from bona fide NPCs at each embryonic stage.[6] This finding indicates that the potential of NPCs decreases with embryonic development, irrespective of their history (despite no production of neurons).

Upon binding to a ligand such as Delta-like1, the cleaved intracellular domain of the Notch receptor (NICD) enters the nucleus.[7] NPCs receive essential signals for their maintenance, probably Notch ligands, from BPs and newborn neurons, which are positive for Mindbomb homolog 1 (Mib1).[9] Mib1 is required for the endocytosis of the Notch ligands.[10]

NICD cooperates with the DNA-binding protein CBF1 (also known as RBPJ) and its coactivator Mastermind like (Maml) to activate *Hes1*, *Hes5* and *Hes*-related genes, *Hey1* and *Hey2*, all of which encode Hes-type basic helix-loop-helix (bHLH) transcription factors.[11,12] Hes1 forms homodimers as well as heterodimers with Hey1 or Hey2 and the dimers bind specific sequences, including a *Mash1* (also known as *Ascl1*) promoter, to repress the gene.[12,13] Transfection of either one of the above *Hes* and *Hey* genes can repress transcription of proneural genes such as *Neurogenin 2* (also known as *Neurog2* or *ngn2*) and *Mash1*, which are crucial for neuronal differentiation, thereby maintaining NPCs.[12-14]

Conversely, loss-of-function mutants of the Notch pathway, such as $Notch1^{-/-}$ and $cbf1^{-/-}$ mice, display precocious neuronal differentiation and NPC depletion.[11] Similar phenotypes are observed only in limited portions of the developing nervous system in single mutants of *Hes* genes,[12] indicating that *Hes* and *Hey* genes are functionally redundant in most of the nervous system. In the forebrain, many NPCs are normally maintained even in triple mutants of *Hes1*, *Hes3* and *Hes5*.[15] Because $Notch1^{-/-}$ and $cbf1^{-/-}$ mice cannot survive until the neocortex is formed, the function of the Notch pathway has been further determined using conditional knockout mice.[16] Tamoxifen-induced conditional knockout mice of *cbf1* exhibit precocious neuronal differentiation and depletion of nearly all NPCs in the developing neocortex, suggesting that CBF1 may be a nonredundant factor in the Notch pathway for the development of the neocortex.

Although Hes-type proteins are essential effectors of the Notch pathway, whether there are other types of effectors is largely unknown. Injection of a γ-secretase inhibitor, L-685,458, which blocks cleavage and activation of Notch,[17] causes precocious neuronal differentiation and NPC depletion in the developing neocortex, concomitant with *Hes5* repression.[18] Depletion of NPCs is not completely suppressed by cotransfection of *Hes1*, *Hes5*, *Hey1* and *Hey2* (see Fig. 3),[18] suggesting that there may be another essential effector of the Notch pathway. Furthermore, incomplete suppression by the *Hes* and *Hey* genes is also observed when the Notch pathway is blocked by a dominant negative form of Maml1 (DN-Maml1).[18]

IDENTIFICATION OF NEPRO

Using Digital Differential Display, we have identified genes specifically expressed at early stages in the developing neocortex. One of the genes is named *Nepro*, because it is necessary for the maintenance of NPCs.[18] *Nepro* is expressed only at early stages (E9.5 to E14.5) in the VZ of the forebrain, which is occupied by many NPCs.

Nepro encodes a 564-amino acid protein that contains no other known structural motif except a nuclear localization signal (NLS). This protein is thought to be a nuclear

protein, because a Nepro protein fused with a peptide tag is localized in the nucleus of NPCs. Unlike Hes-type proteins, Nepro does not contain a bHLH domain. There is a single *Nepro* homolog in each vertebrate species but no *Nepro* homolog in invertebrates. All the Nepro proteins have three conserved regions: QVEQC, a hydrophobic amino acid-rich region and DDIDDIF (Fig. 2A).

NEPRO INHIBITS NEURONAL DIFFERENTIATION AND IS NECESSARY FOR THE MAINTENANCE OF NPCs

The function of *Nepro* has been examined by transfecting it into the developing neocortex using in vivo electroporation.[19,20] Transfection of *Nepro* at E13.5 inhibits neuronal differentiation and maintains NPCs, similar to that of of *Hes1* and *Hes5* (Fig. 2B, upper panel). Nepro-expressing cells continue to proliferate and remain as NPCs in the VZ, as shown by labeling of the proliferation marker Ki67 and the NPC marker Nestin. Expression of *Neurogenin2* and *Mash1* is also repressed by *Nepro* as well as *caNotch*, *Hes1* and *Hes5*.

Interestingly, transfection of *Nepro* at later stages does not affect NPCs, whereas *caNotch*, *Hes1* and *Hes5* inhibit neuronal differentiation and maintain NPCs at both early and later stages (Fig. 2B, lower panel).

Knockdown of *Nepro* and transfection of a truncated dominant negative form of *Nepro* (Δ*Nepro*) lead to precocious neuronal differentiation and NPC depletion (Fig. 2C), indicating that *Nepro* is required for maintaining NPCs.

NEPRO IS IN PARALLEL WITH *HES*, DOWNSTREAM OF NOTCH

Both gain- and loss-of-function experiments of the Notch pathway have indicated that *Nepro* expression is activated downstream of Notch. *caNotch* transfection activates *Nepro*. On the other hand, *Nepro* is repressed by blocking the Notch pathway with injection of L-685,458, *cbf1* knockdown or *DN-Maml1* transfection.

Nepro is neither downstream nor upstream of *Hes* genes. Whereas transfection of *Hes1* and *Hes5* does not change *Nepro* expression, expression of *Hes1* and *Hes5* is unaffected by *Nepro* transfection or knockdown.

NEPRO IS AN ESSENTIAL EFFECTOR FOR THE NOTCH PATHWAY

The activity of *caNotch* to inhibit neuronal differentiation and maintain NPCs is completely blocked by *Nepro* knockdown or Δ*Nepro* transfection, indicating that *Nepro* is necessary for the function of the Notch pathway.

Moreover, when Notch activity is blocked by L-685,458 or DN-Maml1, cotransfection of *Hes1*, *Hey1* and *Nepro* is sufficient to inhibit neuronal differentiation and maintain NPCs, whereas contransfection of *Hes1, Hes5*, *Hey1* and *Hey2* is not (Fig. 3). These findings indicate that Nepro is a novel Notch effector and that the combination of Nepro and Hes-type proteins is crucial for maintaining NPCs in the developing neocortex.

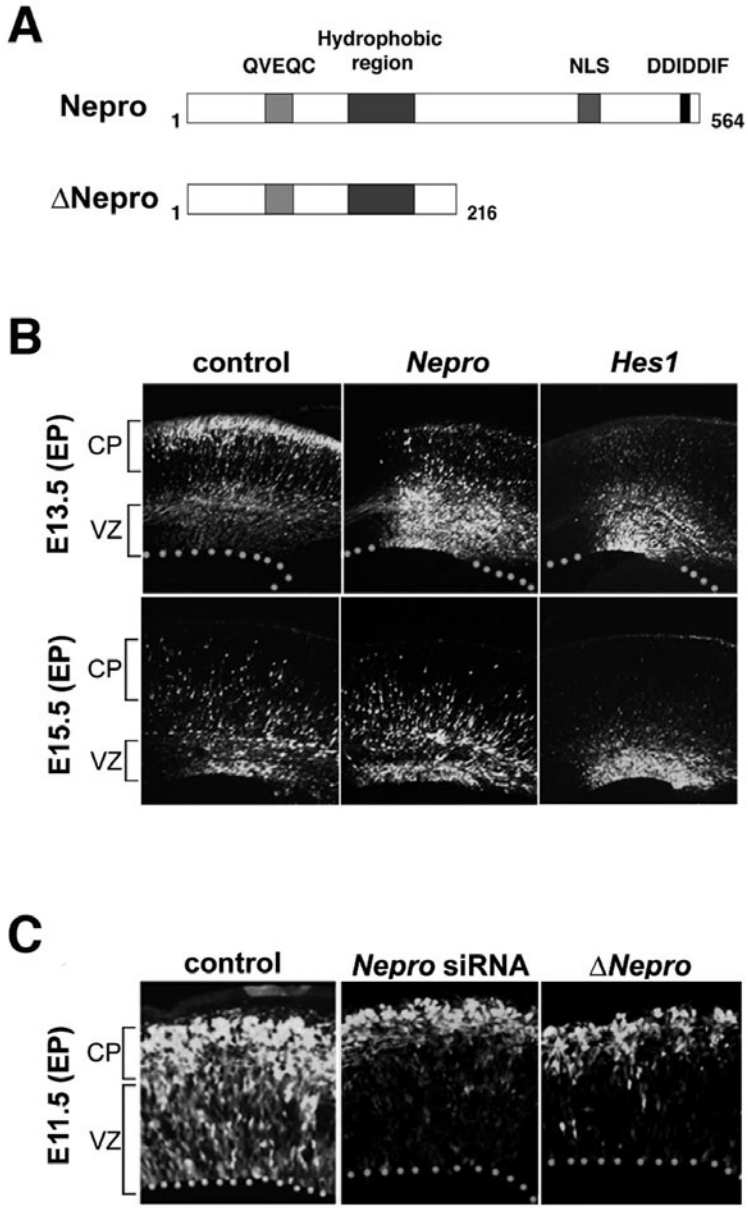


Figure 2. A) Structure of the Nepro protein and a deletion mutant (ΔNepro). B) NPCs in the neocortex are transfected with *enhanced yellow fluorescent protein* (*EYFP*) as a control or cotransfected with *EYFP* and *Nepro* or *Hes1*, using in vivo electroporation (EP). Transfection of *EYFP* shows normal development (proper proportion of NPCs in the VZ and differentiating neurons in the CP). Transfection of *Nepro* inhibits neuronal differentiation and maintains NPCs at early stages (E13.5) but not at later stages (at E15.5). Hes1 inhibits neuronal differentiation and maintains NPCs at both early and later stages. C) In contrast to normal development by *EYFP* as a control, repression of *Nepro* by its siRNA or Δ*Nepro* transfection causes precocious neuronal differentiation and NPC depletion. Reprinted from Muroyama Y, Saito T. Development 2009; 136:3889-3893.

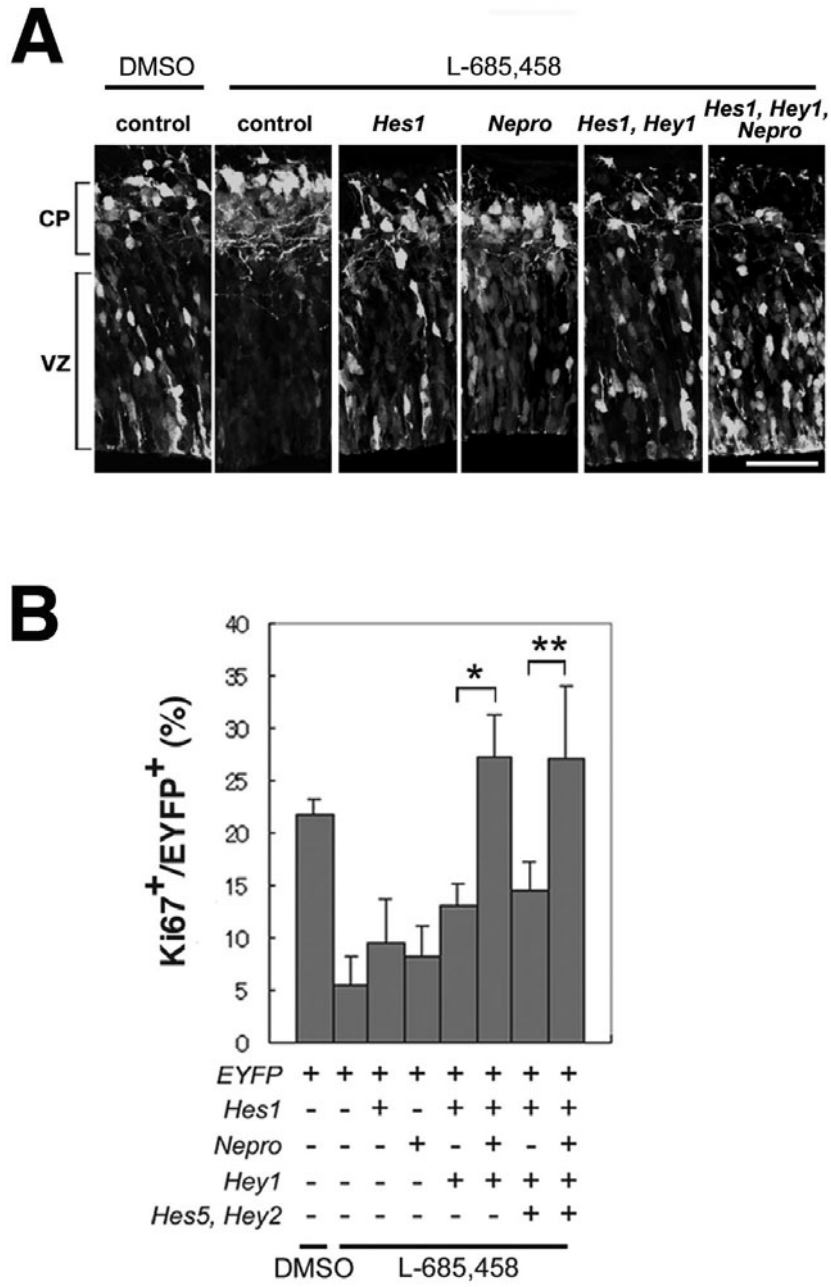


Figure 3. Combination of Nepro and Hes-type proteins maintains NPCs when the Notch pathway is blocked. *EYFP* transfection shows normal development in the presence of a solvent, DMSO, as a control. Injection of L-685,458 causes precocious neuronal differentiation and NPC depletion. Combined transfection of *Hes1*, *Hey1* and *Nepro* is sufficient to maintain NPCs ($Ki67^+$ cells), whereas any combination without *Nepro* is insufficient. *P < 0.001, **P < 0.005. Scale bar: 50 μm. Reprinted from Muroyama Y, Saito T. Development 2009; 136:3889-3893.

DISCUSSION

Functional analyses of Nepro have revealed that the Notch pathway is bifurcated downstream of Notch in the developing neocortex (Fig. 4). *Nepro* may be directly activated by the NICD-CBF1-Maml complex, because there is a CBF1-binding site upstream of *Nepro*.

Because transfection of *Nepro* at later stages has no effect in contrast to *Hes1* and *Hes5*, Nepro may require a partner protein that is expressed only at early stages. The partner protein will be different from Hes-type proteins, because they are expressed at both early and later stages. The dominant negative activity of ΔNepro suggests that Nepro bridges at least two factors (Fig. 5A,B). Although both Nepro and Hes-type proteins are necessary, it is less likely that Nepro directly associates with Hes-type proteins, because ΔNepro does not affect NPCs at later stages when *Hes* genes are expressed. There are several possibilities regarding Nepro functions. (1) The complex containing Nepro may associate with or modify Hes-type proteins (or a complex containing Hes-type proteins). (2) The Nepro complex may create a condition in which Hes-type proteins (or the complex containing Hes-type proteins) become active, such as modification of chromatin structure. (3) Factors that are produced downstream of Nepro may interact with or modify Hes-type proteins (or the complex containing Hes-type proteins). At later stages of neocortex development, another factor may substitute for Nepro (Fig. 5C), or Nepro may become unnecessary once Hes-type proteins (or the complex containing Hes-type proteins) are active. Therefore, it will be important to determine the factors that interact with Nepro.

The temporally restricted expression and function of Nepro indicates that there are two stages involved in the maintenance of NPCs in the developing neocortex, Nepro-dependent early stages and Nepro-independent later stages. NPCs change their character, e.g., gradual restriction of their potential, in the developing neocortex. The

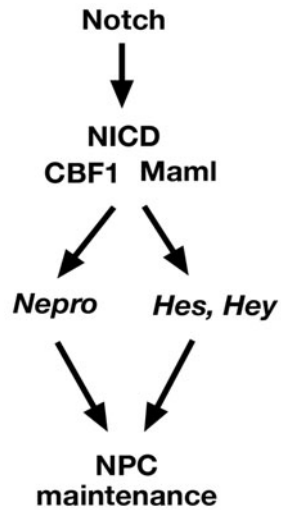


Figure 4. The Notch pathway in the developing neocortex at early stages.

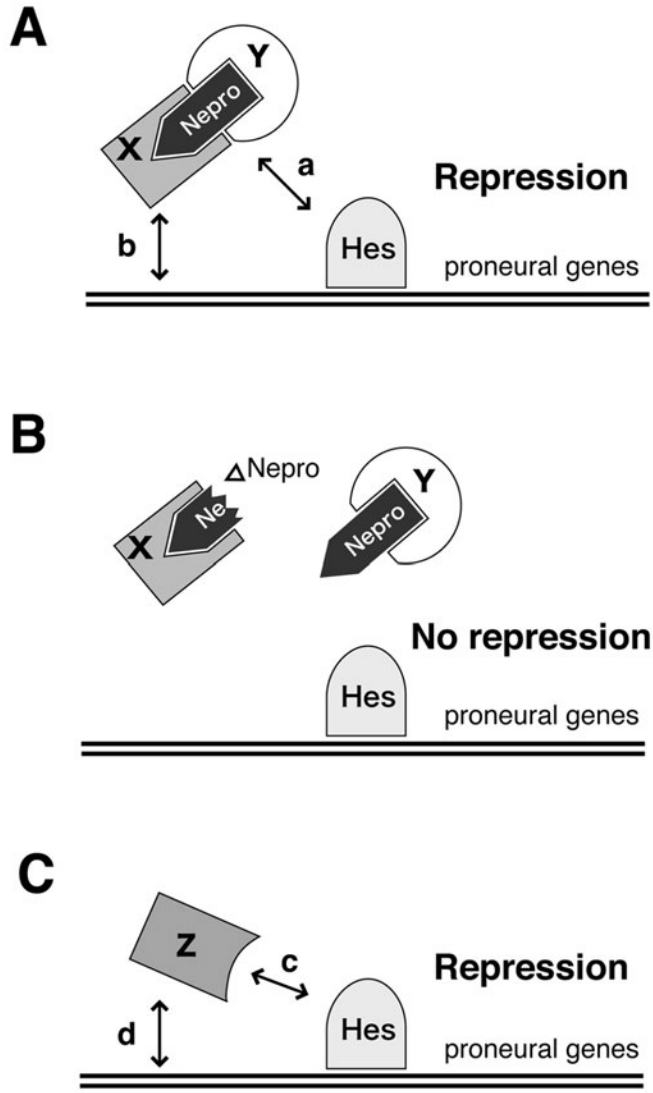


Figure 5. Schematic illustration of the possible functions of Nepro. A) A complex composed of Nepro and two other factors (X and Y) may interact with or modify Hes-type proteins (or a complex containing Hes-type proteins) to repress proneural genes (a). The Nepro complex may make a condition for the function of Hes-type proteins (b). B) ΔNepro blocks the Notch pathway, suggesting that Nepro bridges the two factors. C) Another factor (Z) may substitute for Nepro function (c or d) at later stages when Nepro is absent.

mechanism that controls the temporal character of NPCs is unknown, but it is suggested to be intrinsic to NPCs because isolated NPCs are able to sequentially generate several types of neurons in vitro in the same order as in vivo.[21] It remains to be determined whether Nepro is involved in specification of the temporal character of NPCs.

Although most components of the Notch pathway are conserved throughout vertebrates and invertebrates,[22] no invertebrate homolog of *Nepro* has been found. Amino acid sequences of Nepro are well conserved among primates (more than 94% identity), similar to those of Notch1 and Hes1. In contrast, Nepro is less conserved between other species, i.e., human to mouse (65%), frog (35%) and zebrafish (28%), whereas Notch1 and Hes1 are more conserved, i.e., human to mouse (91%, 93%), frog (71%, 67%) and zebrafish (71%, 53%) for Notch1 and Hes1, respectively. Nepro may be a key factor for the development of the neocortex, because it is expressed and functional in the developing neocortex, which is highly developed in mammals, particularly in primates. Nepro may be also functional in other tissues, because species that do not share the six-layered neocortex structure, such as frog and fish, also possess Nepro.

CONCLUSION

Nepro is an essential Notch effector for the maintenance of NPCs in the developing neocortex at early stages. Nepro molecular functions and the factors that may substitute for Nepro in the developing neocortex at later stages and in other tissues are yet to be determined.

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CHAPTER 6

NOTCH SIGNALING AND DEVELOPMENT OF THE HEMATOPOIETIC SYSTEM

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Abstract: Notch signaling exerts multiple important functions in the hematopoietic system. Notch1-mediated signals are essential to induce the onset of definitive hematopoiesis within specialized domains of hemogenic endothelium in the fetal dorsal aorta. In contrast, Notch is dispensable for the subsequent maintenance of hematopoietic stem cells in the adult bone marrow. Notch is a key regulator of early T-cell development in the thymus. An expanding number of hematopoietic and lymphoid cell types have been reported to receive context-dependent inputs from the Notch pathway that regulate their differentiation and function. Progress in the field will continue to bring fundamental information about hematopoiesis and practical insights into the potential to modulate Notch signaling for therapeutic purposes.

INTRODUCTION

Although Notch signaling plays an important role in multiple organs and tissues, the attention of the scientific community was first attracted to this pathway in mammalian organisms through a link between Notch and the hematopoietic system. Indeed, the existence of human NOTCH was discovered on the basis of a t(7:9) translocation in acute T-cell lymphoblastic leukemia (T-ALL) that revealed involvement of the *NOTCH1* gene on chromosome 9.[1] The t(7:9) rearrangement drove expression of a truncated and constitutively active oncogenic NOTCH1 protein.[1,2] This initial work was followed by a period of rapid discovery during which the four mammalian Notch receptors (Notch1-4) were identified and biochemical principles of Notch activation were characterized (for a review, see refs. 3, 4). Since then, a large body of work has been performed to define the

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role of Notch signaling in normal hematopoiesis and its dysregulation in T-cell leukemia (for a review, see ref. 5-10). Progress in the field benefits from scientific information derived from multiple organisms, including zebrafish, mice and humans, revealing a high degree of conservation during evolution. Here, we discuss our current understanding of Notch signaling in hematopoietic development, with a focus on its essential function in the emergence of definitive hematopoietic stem cells (HSCs) and its effects at subsequent stages of hematopoiesis.

OVERVIEW OF NOTCH SIGNALING

Notch signaling is a highly conserved cell-cell communication pathway that converts the intracellular domain of a cell surface receptor into a transcriptional activator via regulated intramembrane proteolysis (Fig. 1).[3,4] Flies have a single *Notch* receptor gene and two Notch ligand genes called *Serrate* and *Delta*. Mammals possess four Notch receptor genes (*Notch1-4*) encoding related proteins, as well as five well-documented Notch ligand genes that belong to the *Jagged* and *Delta-like* families (*Jag1, Jag2, Dll1, Dll3, Dll4).* The agonistic nature of Dll3 has been questioned and it is possible that it acts as a natural antagonist of Notch signaling.[11]

The expression of Notch receptors and ligands is highly regulated. In the hematopoietic system, this dynamic regulation favors interactions between specific receptors and

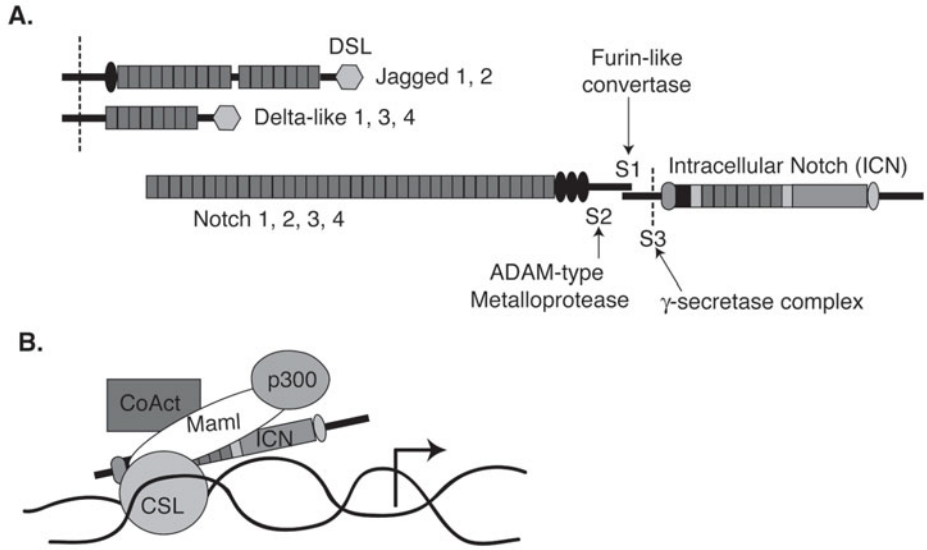


Figure 1. Canonical Notch signaling. A) Notch signaling occurs upon interaction between one of five ligands from either the Jagged (1, 2) or Delta-like (1, 3, 4) families and one of four Notch receptors (Notch1-4). Notch receptors are cleaved in the Golgi complex by a furin-like convertase (S1), leading to expression of a heterodimeric Notch receptor. Upon ligand binding, the S2 cleavage site is made accessible to an ADAM-type metalloprotease. The last cleavage (S3) is mediated by the γ-secretase complex and releases the Notch intracellular domain (ICN). B) ICN translocates into the nucleus to interact with the DNA binding protein, CSL (encoded by the *Rbpj* gene), a member of the Mastermind-like (Maml) family and other transcriptional coactivators. DSL: Delta/Serrate/Lag-2 domain.

ligands in different specialized microenvironments (e.g., Dll4 and Notch1 for developing thymocytes, Dll1 and Notch2 for splenic marginal zone B-cells).[12-15] In addition, ligands may differ biochemically in their affinity for specific receptors or in their ability to trigger receptor activation. To date, the mechanistic basis for these differences is not understood in detail, in part because very limited structural information is available as to how the extracellular domains of Notch ligands and receptors interact. However, it is clear that posttranslational modifications of the Notch receptors by Fringe family glycosyltransferases can alter the relative binding affinity of ligand-receptor partners or the efficiency of receptor activation.[16-19] For example, Dll4 displays a preferential interaction with the Fringe-modified Notch1 receptor in several contexts, including during early T-cell development and angiogenesis.[17,20,21] This complex regulation can create a paradoxical situation in which Jagged and Delta-like ligands act as Notch agonists when expressed in isolation, but Jagged can antagonize Delta-like ligands when both are present.[20] This is a situation in which Jagged family members can be described in pharmacological terms as partial agonists.

Notch proteins are expressed on the cell surface as heterodimeric Type I transmembrane receptors, after constitutive cleavage at an extracellular "S1" site during transport through the Golgi complex.[22] After ligand binding, Notch receptors are activated by regulated intramembrane proteolysis.[23] Notch receptors are first cleaved at an extracellular "S2" site close to the transmembrane domain by an ADAM family metalloprotease.[24,25] In the hematopoietic system, the ADAM10 protease was recently identified genetically as the critical enzyme mediating this cleavage, at least for ligand-mediated proteolysis of Notch1 in developing T-cells and Notch2 in splenic B-cells.[26,27] Crystallographic data indicate that, in the absence of signaling, the S2 cleavage site remains hidden within a hydrophobic pocket in the Negative Regulatory Region (NRR) of the receptor.[28,29] Current models support the idea that ligand binding destabilizes the NRR and allows ADAM10 to access its target site in the receptor. Interestingly, recurrent point mutations identified in human and mouse T-cell leukemia destabilize this region, creating a constitutively active *Notch1* allele that is cleaved even in the absence of ligand.[7,8,30] The NRR of Notch1, Notch2 and Notch3 has recently been targeted successfully by specific monoclonal antibodies that stabilize this region even upon ligand binding, preventing S2 cleavage and subsequent activation of the receptor.[31,32]

Cleavage at the S2 site generates an unstable intermediate that becomes a target for intramembrane proteolysis by gamma secretase (S3 site).[33] Gamma secretase is a large multiprotein complex that mediates regulated proteolysis of several transmembrane proteins.[23,34-36] This rate-limiting step in Notch activation can be blocked by gamma secretase inhibitors.[37] After gamma secretase-mediated proteolysis, the intracellular domain of the Notch receptor (ICN) is released into the cytoplasm and migrates into the nucleus, where it binds the transcription factor CSL/RBP-Jk and becomes part of a large transcriptional activation complex (Fig. 1B).[38-41] This process has been referred to as "canonical Notch signaling" when compared to alternative mechanisms of signaling that have been hypothesized to exist, in particular in Drosophila. Of note, all well-defined effects of Notch signaling in mammalian organisms have so far been ascribed to canonical signaling via this transcriptional complex.

In the absence of Notch signaling, CSL/RBP-Jk is recruited to target gene sequences in association with a repressor complex. Although the functional importance of the repressor complex remains debated, its composition is being elucidated, with recent data revealing the presence of the corepressor MINT/SHARP and the histone demethylase

KMD5A.[42-44] Upon Notch activation, ICN binds CSL/RBP-Jk and recruits a member of the Mastermind-like family (MAML1-3) of transcriptional coactivators.[45-47] MAMLs possess a conserved N-terminal alpha-helix that binds a composite interface formed by CSL/RBP-Jk and ICN.[41] MAMLs recruit additional coactivators into the complex and this function is essential for efficient Notch-mediated transcriptional activation. Peptides or recombinant proteins containing only the N-terminal MAML region act as potent and specific dominant negative inhibitors of Notch signaling.[48-50]

The nature and spectrum of Notch target genes in different tissues remain incompletely understood. Members of the Hairy/Enhancer-of-split (*Hes*) and Hairy-related (*Hrt* or *Hey*) families have been well described as Notch target genes in multiple contexts.[4] However, they do not account for the effects of Notch signaling in all situations. Furthermore, regulatory inputs other than Notch signaling participate in the transcriptional activation of these genes.[51-53] Thus, changes in the expression of these genes do not necessarily result from Notch activity. Another important open question in the field is whether different Notch family members (Notch1-4) or different MAML coactivators (MAML1-3) generate transcriptional activation complexes that have different properties. Although genetic evidence suggests that this is the case, the mechanistic basis for these effects remains to be elucidated. In addition, it is possible that ICN can interact with unusual and perhaps tissue-specific transcriptional partners (e.g., CREB, SMAD), a phenomenon that could increase the diversity of functional outputs that result from Notch activation.[54,55]

In the hematopoietic system, Notch is used recurrently for different functions at various stages of development (Fig. 2). Both in zebrafish and in mice and presumably in humans, Notch is absolutely required for the emergence of definitive HSCs during mid-gestation, while dispensable for primitive hematopoiesis.[56-58] At subsequent stages of development, Notch plays an essential role in several hematopoietic and lymphoid lineages, in particular at early stages of T-cell development and during the differentiation of splenic marginal zone B-cells.[12,14,59-61] Additional functions have been reported in megakaryocyte development, in dendritic cell lineages and in peripheral B- and T-cell differentiation.[62-66] However, Notch appears dispensable in multiple other lineages, including myelo-erythroid cells and bone marrow B-cells and it is not required at

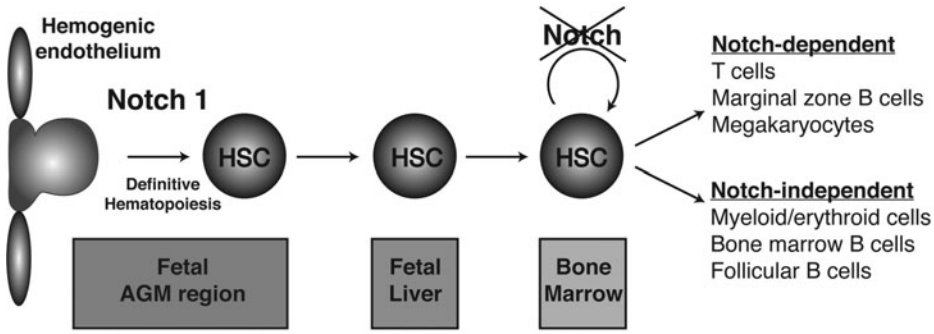


Figure 2. Overview of Notch signaling in hematopoiesis. Notch1 is absolutely required for the onset of definitive hematopoiesis in the aorta-gonad-mesonepheros (AGM) region. However, Notch signaling is dispensable for primitive hematopoiesis and for maintenance of adult hematopoietic stem cells. At later stages of hematopoiesis, Notch is absolutely required for the development of several lineages, including T-cells and marginal zone B-cells, while dispensable in other lineages.

physiological levels of signaling for the self-renewal and maintenance of adult HSCs.[67-69] At least in part, these differential effects are regulated through a tight control of Notch signaling intensity in different hematopoietic compartments, so that certain progenitors only experience a very low intensity of signaling, while others are exposed to a high level of Notch signals. Subsequent sections will discuss these different effects in detail.

NOTCH AND EMERGENCE OF DEFINITIVE HEMATOPOIETIC STEM CELLS

As first observed in birds and subsequently in mammals, hematopoietic development starts in two distinct phases, first at extraembryonic sites (primitive hematopoiesis) and subsequently in the embryo itself (definitive hematopoiesis).[70,71] The primitive wave is initiated in the yolk sack, while the second definitive wave originates mostly from the aorta-gonad-mesonephros (AGM) region, as well as from the placenta.[72,73] Within the AGM, a subset of endothelial cells in the ventral wall of the dorsal aorta has been identified as the site of emergence of definitive HSCs (Fig. 3).[73-75] This so-called hemogenic endothelium, demonstrated in both mice and zebrafish, undergoes a series of morphologic changes to give rise to HSCs. In the mouse, endothelial cells destined to give rise to HSCs bud into the lumen of the dorsal aorta.[73] Upon entering the circulation, HSCs can then home to the developing fetal liver to allow HSC expansion and hematopoiesis. In the zebrafish, recent elegant imaging studies have shown that hemogenic endothelial cells round up and dissolve contacts with neighboring cells, before migrating into the sub-aortic space.[74,75]

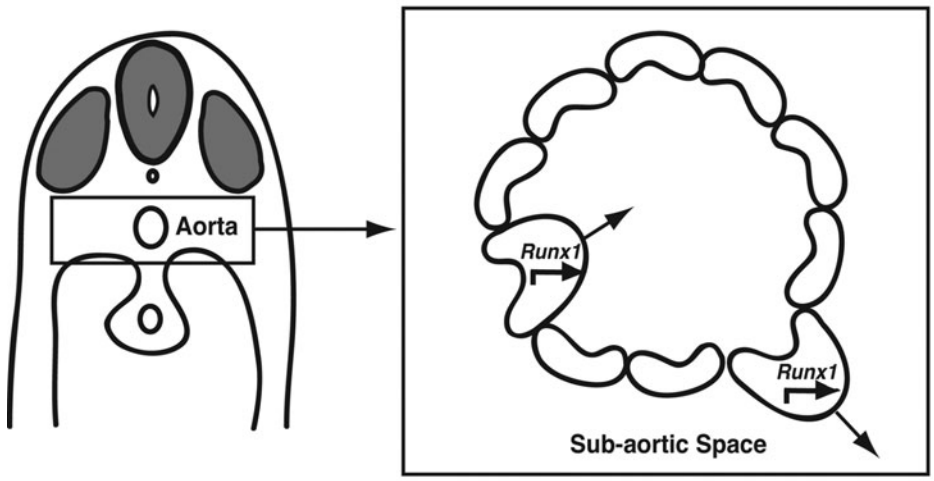


Figure 3. Emergence of definitive HSCs. The ventral wall of the dorsal aorta (inset) within the aorta-gonad-mesonephros (AGM) region of the mid-gestation embryo is lined with a unique hemogenic endothelium. Endothelial cells, following activation of Notch and other signaling pathways, upregulate *Runx1* expression and undergo a specialized transition. In mice, newly formed hematopoietic cells bud into the aortic lumen and enter circulation, eventually homing to the fetal liver. In zebrafish, the hemogenic cells round up, freeing themselves from contacts with neighboring cells and migrate into the sub-aortic space. These cells then enter the axial veins and home to the kidney marrow.

From this location, the newly formed HSCs enter the axial vein and circulate to the kidney marrow, allowing HSC expansion and hematopoietic development.

The definitive wave of hematopoiesis depends on active Notch signaling. Mouse embryos deficient in *Notch1* have normal primitive hematopoietic progenitors, but fail to initiate the definitive program and to specify the emergence of HSCs in the embryo proper.[57] Experiments with blastocyst chimeras have shown that this is a cell-autonomous phenomenon.[56] *Notch1*-deficient fetuses fail to upregulate expression of the transcription factor *Runx1*. *Runx1* is a member of the core binding factor family of transcription factors that functions together with its heterodimeric partner CBFβ and is essential for HSC emergence.[76,77] Runx1 expression is required within a VE-cadherin$^+$ endothelial compartment to support HSC emergence, while dispensable later for HSC maintenance.[78] Functional data suggest that *Runx1* operates downstream of Notch signaling (Fig. 4). Indeed, *Runx1* expression was sufficient to rescue hematopoiesis from *Notch1*-deficient para-aortic splanchnopleural explants.[79] Furthermore, conditional expression of a constitutively active form of Notch increased Runx1 expression and HSC expansion in the zebrafish AGM region.[77] The exact mechanism by which Notch activates *Runx1* is not completely clear, but two possibilities exist: either Notch activates *Runx1* expression directly or it functions through an intermediate factor. This latter possibility is supported by data in which Notch signaling was blocked via inactivation of the *Rbpj* gene (encoding CSL/RBP-Jk).[80] These mice showed reduced association of Notch with the *Gata2* promoter by chromatin immunoprecipitation. This offers a potential explanation for the reduced *Runx1* expression found when Notch signaling is abrogated, as *Gata2* is a known regulator of *Runx1* expression via binding to a *Runx1* enhancer element.[81]

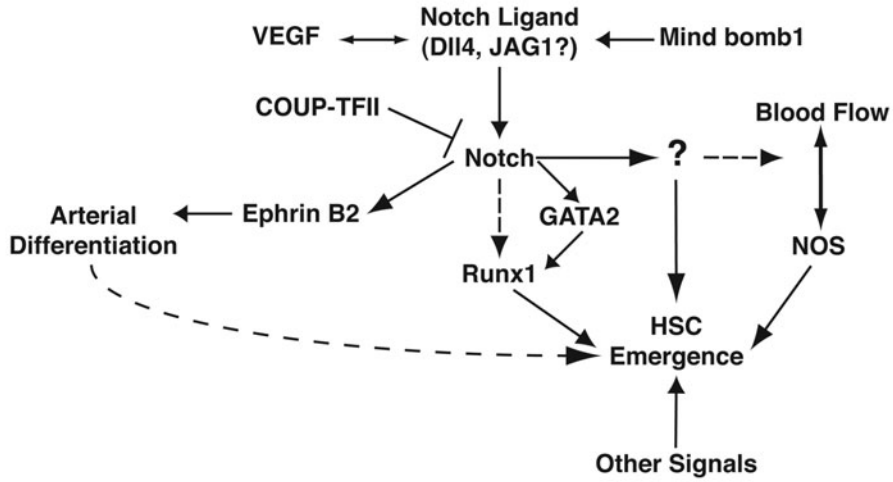


Figure 4. Putative signaling hierarchy centered on Notch in the AGM region. The activation of Notch signaling is intimately associated with both hematopoietic stem cell emergence and vascular development. Notch promotes arterial development through upregulation of *Ephrin B2* expression. Furthermore, expression of the Notch ligand Dll4 is regulated by VEGF and is important in endothelial sprouting. In developing HSCs, Notch signaling may directly or indirectly activate *Runx1* expression, which is essential for HSC emergence. Along with *Runx1*-mediated effects, Notch may impact additional factors affecting this process, including nitric oxide release and blood flow.

However, it remains possible that other unidentified factors connect Notch activation to Runx1 expression (Fig. 4).

The intimate link between Notch signaling and vascular development has complicated the understanding of Notch's role in the emergence of definitive HSCs. Indeed, *Notch1*-deficient mice and, even to a greater extent, mice deficient in both *Notch1* and *Notch4* have profound defects in early vascular development and die *in utero* before E10.5.[82] Importantly, these findings are recapitulated by conditional *Notch1* inactivation with a *Tie2-Cre* transgene.[83] Thus, Notch1 plays an essential role in vascular precursors and/or their endothelial progeny expressing the tyrosine kinase receptor Tie2 (also known as Tek). In addition, Notch signaling regulates vascular sprouting, an essential component of angiogenesis. Vascular branching requires that an endothelial bud, known as the tip cell, migrates ahead of a stalk of proliferating endothelium to generate a new vessel. Dll4 signaling through Notch1 restricts this process to control tip cell development and ensure normal vascular patterning.[20,84,85] Furthermore, Notch also plays an important role in specifying arterial versus venous differentiation.[86,87] Notch regulates expression of the ligand Ephrin B2 in arterial vessels. The interaction of Ephrin B2 and its receptor EphB4 in venous endothelium is essential for normal arterial and venous development.[88] Findings in mouse and zebrafish models suggest that Notch signaling is required to establish Ephrin B2 expression and arterial identity while suppressing venous potential.[86,87] In addition, Notch signaling is actively suppressed at venous sites via the transcription factor COUP-TFII.[89] Since the hemogenic endothelium in the dorsal aorta is a key site of definitive hematopoiesis, one can imagine that failure of arterial differentiation could impede HSC emergence independently of HSC-specific Notch signals. Thus, are the effects of Notch signaling on HSC specification independent of its effects in vascular development and arterial versus venous differentiation?

Shedding some light on this question, Bigas and collaborators have reported that the Notch ligand Jagged1 is required for the emergence of normal numbers of definitive hematopoietic cells from the AGM region in a Gata2-dependent manner, while being dispensable for arterial development.[90] Mice deficient in Jagged1 had markedly reduced numbers of Ly6A-GFP$^+$ hematopoietic progenitors in the E10.5 AGM tissues. This defect could be rescued ex vivo either by coculturing AGM cells with OP9 stromal cells expressing *Jag1* or by expressing *Gata2* via lentiviral transduction. Nevertheless, Jagged1-deficient mice demonstrated normal arterial development. Taken together, these data suggest that *Jag1* is an important ligand to induce Notch signaling in the AGM, independently of the effects that the pathway exerts on arterial specification. In support of this idea, aortic identity was shown not to require *Runx1* expression.[77] These data suggest that Notch signaling modulates two distinct developmental programs: arterial specification and HSC emergence. While there may be some overlap in the machinery involved in these events, further work will be required to elucidate the molecular basis for this distinction and define signals that may be upstream and downstream of Notch signaling in these two specific contexts.

Besides *Notch1* or *Rbpj* inactivation, which block Notch signaling in the signal-receiving cells, mouse and zebrafish *mind bomb 1* mutants fail to generate definitive HSCs.[77,91] *Mind bomb 1* encodes an E3 ubiquitin ligase that plays an essential role in cells expressing Notch ligands by promoting their capacity to trigger efficient Notch signaling. This occurs via mechanisms that remain to be fully clarified but likely involve endocytosis of the Notch ligands.[92] The identity of the Notch ligands that mediate Notch signaling in the AGM region is incompletely understood. Jagged1, Jagged2, Dll1 and

Dll4 were reported to be expressed in the AGM region.[90,91] As discussed previously, Jagged1 but not Jagged2-deficient mice display a profound impairment in the emergence of HSCs.[90] However, this does not rule out an important function for Dll1 and/or Dll4. Dll4 in particular is difficult to investigate since it interacts genetically with VEGF signaling and loss of even one *Dll4* copy leads to embryonic lethality with massive vascular defects.[93] Conditional genetic approaches will be necessary to investigate further the role of *Dll4* in the AGM region. This will be essential to identify the cellular partners in the Notch ligand-receptor interaction and the upstream signals that regulate induction of Notch signaling.

Recent evidence indicates that blood flow is a crucial regulator of definitive HSC emergence. Zebrafish carrying a *silent heart* mutation, which prevents the initiation of a heartbeat, demonstrate severely reduced numbers of definitive HSCs associated with reduced *Runx1* expression.[94] Interestingly, these mutant fish have expanded venous differentiation and reduced arterial differentiation in addition to the HSC phenotype, a constellation of phenotypes reminiscent of development in the presence of a Notch signaling deficiency. The effects of blood flow appear at least in part related to activation of nitric oxide (NO) synthase 1. In addition, nitric oxide donors were able to rescue the hematopoietic phenotype of *mind bomb* mutant zebrafish, which lack Notch signaling, suggesting that NO functions downstream of Notch during HSC specification. Future studies will need to determine the molecular mechanisms of this interaction.

In addition to blood flow, prostaglandin E2 (PGE2) seems to play a role in stem cell emergence upstream of *Runx1* expression and therefore potentially upstream of Notch signaling. Zebrafish models utilizing knockdowns of either cyclooxygenases 1 and 2 or PGE2 synthase result in a reduction in stem cells formed from the AGM region.[95] In contrast, administration of PGE2 can enhance stem cell numbers and has been linked genetically to Wnt signaling.[96] These data suggest a putative tie between Notch, PGE2 and Wnt with respect to HSC emergence, but further studies are necessary.

Additional studies will be required to fully map Notch signaling within the regulatory network controlling Runx1 expression and HSC specification in the AGM. This will include identifying the specific Notch receptors and ligands driving Notch signaling in the hemogenic endothelium, defining the cell types that express Notch ligands and receptors in this interaction, understanding how upstream signals trigger Notch signaling and connecting Notch-mediated transcriptional activation to downstream effects, including Runx1 expression. In addition, progress is being made to define the signals that specify the ventral wall of the dorsal aorta as a region with hemogenic potential. For example, BMP4 signaling was recently shown to be critical for patterning of the floor of the dorsal aorta, begging the question as to how it cooperates with Notch signaling in this area.[97] Further work in this field will not only answer interesting developmental questions, but also provide important information about the pathways that need to be considered to derive definitive HSCs from embryonic or induced pluripotent stem cells.

NOTCH AND MAINTENANCE OF HEMATOPOIETIC STEM CELLS

Although it is clear that Notch plays an essential role at the emergence of definitive HSCs, whether it is important to support their subsequent maintenance has been a more controversial question. Early work in the field was influenced by observations

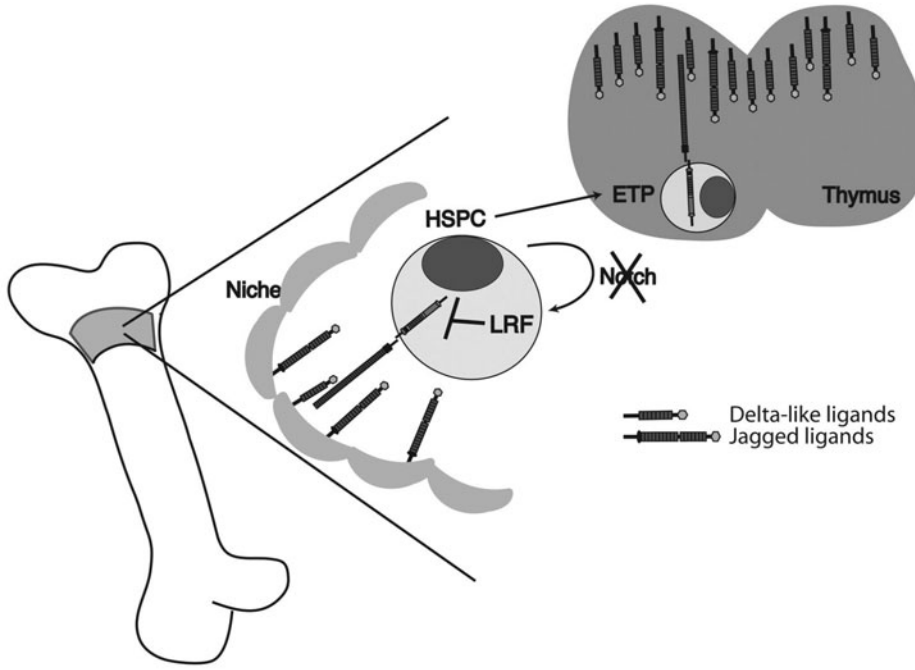


Figure 5. Notch signaling in adult hematopoietic stem cells. Although various elements of the Notch pathway are expressed in the bone marrow, hematopoietic stem/progenitor cells (HSPCs) only experience low levels of Notch signaling. Notch is dispensable for the maintenance of adult HSCs and is actively inhibited by leukemia/lymphoma related factor (LRF). Conversely, upon migration to the thymus, early T lineage progenitors (ETPs) respond to a high density of Dll4 Notch ligands, overcoming LRF-mediated inhibition and driving T-cell development.

in *Drosophila*, where Notch had been described to restrict differentiation of neural progenitors during asymmetric cell division.[98-100] Thus, it was tempting to speculate that Notch would play an analogous role in mammalian stem cells. In this context, Notch receptors were found to be expressed in hematopoietic progenitors, while Notch ligands were identified in the bone marrow microenvironment (Fig. 5).[101-105] A variety of gain-of-function strategies were then used to evaluate the potential of Notch signaling to preserve or expand hematopoietic progenitor populations. This included exposing bone marrow progenitors to a high density of Notch ligands in culture, expressing constitutively active forms of intracellular Notch, overexpressing the Notch target gene *Hes1*, genetically altering osteoblasts and more recently coculturing HSCs with endothelial cell lines that spontaneously express Delta-like Notch ligands.[106-114] All these approaches revealed a significant impact of increased Notch signaling on hematopoietic progenitors, resulting in significant in vitro expansion. This approach is now being explored in a translational setting by the Seattle group to achieve progenitor expansion for adult patients undergoing cord blood transplantation, using ex vivo cultures with recombinant Notch ligands.[114] This is an interesting therapeutic approach since the dose of hematopoietic progenitors is frequently limiting in this setting, resulting in delayed hematopoietic reconstitution.

In contrast to these findings with supraphysiological levels of Notch signaling, most studies using loss-of-function approaches have failed to reveal a requirement for canonical Notch signaling in the maintenance of adult HSCs. Conditional inactivation of *Notch1* and *Jag1* in the bone marrow had no detectable effect on HSCs, at least in steady-state conditions.[69] However, compensatory effects of other Notch receptors and ligands could not be ruled out in this study. To address these questions, HSC function was studied upon blockade of Notch signaling downstream of all Notch receptors using the pan-Notch inhibitor DNMAML or CSL/RBP-Jk deficiency.[67,68] These studies demonstrated normal maintenance of Notch-deficient HSCs, both in steady-state conditions and in situations of hematopoietic stress, such as after irradiation and HSC transplantation. Secondary transplantation also failed to reveal a subtle defect in the function of Notch-deprived HSCs.[68] These findings differ from other in vivo observations using a dominant negative inhibitor of Xenopus CSL/RBP-Jk for reasons that remain to be determined.[115] Altogether, the bulk of experimental work using well-characterized genetic approaches indicates that Notch is not a major player in the regulation of HSC maintenance.

Given these observations, it was important to evaluate how much Notch signaling HSCs normally experience in the bone marrow. In fact, known Notch target gene transcripts such as *Hes1* or *Dtx1* are present only in very low abundance in primitive hematopoietic progenitors containing HSCs, when compared for example to their progeny in the thymus that experience high levels of Notch signaling.[68,116] This was surprising given the presence of functional Notch receptors in HSCs and Notch ligands in the bone marrow microenvironment. An interesting explanation for this paradox was provided by Maeda and colleagues when they characterized the hematopoietic phenotype of mice lacking the transcriptional repressor LRF/Zbtb7.[116] These mice displayed aberrant extrathymic T-cell development and suppression of B-cell development in the bone marrow, a phenotype reminiscent of Notch gain-of-function studies.[117] Indeed, Notch signaling is triggered at very high levels in LRF-deficient primitive hematopoietic progenitors, via a cell-autonomous mechanism.[116] The molecular mechanisms of this interaction remain to be characterized, but these findings indicate that Notch signaling is actively suppressed by LRF/Zbtb7 when HSCs reside in the bone marrow. Why this is the case is a matter of speculation, but preservation of the B-cell lineage is one of several potential explanations. In any case, these observations are useful to explain the discrepant findings in gain and loss-of-function Notch models with regard to HSC function, since low physiological levels of Notch signaling may not result in detectable functional changes upon Notch inhibition.

Collectively, these data demonstrate that Notch signaling is essential for the emergence of definitive HSCs during fetal life, but dispensable for their subsequent maintenance in vivo. However, this does not rule out a potential role for Notch signaling during certain specific situations of hematopoietic stress in which Notch signaling intensity may be upregulated. This includes effects of Notch signaling after chemotherapy-mediated ablation and at early stages after stem cell transplantation. In addition, Notch may have important functions for nonhematopoietic elements of the bone marrow, such as mesenchymal progenitors, endothelial cells or osteoblasts.[113,118,119] These effects in turn could influence the function of the HSC niche and HSCs via non-cell-autonomous mechanisms. The cumulative impact of all these effects will have to be considered to develop safe methods of therapeutic Notch inhibition in vivo.

NOTCH AND T-CELL DEVELOPMENT

The best characterized function of Notch signaling in the hematopoietic system is its requirement at early stages of T-cell development in the thymus (Fig. 6). The first studies addressing this question used a combination of complementary loss-of-function and gain-of-function models: mice lacking Notch1 in hematopoietic progenitors completely failed to generate T-cells,[12] while constitutively active forms of Notch induced extrathymic T-cell development and suppressed B lineage cells in the bone marrow.[117] Since then, multiple studies have confirmed these findings and identified the specific components of the Notch pathway that mediate these effects. Signaling is triggered by the interaction of Notch1 in thymus-seeding progenitors with the Notch ligand Dll4 expressed in thymic epithelial cells.[12,13] This interaction is modulated by the Lunatic Fringe glycosyltransferase, resulting in enhanced interaction between Dll4 and Notch1 upon Fringe-mediated modification of the Notch1 extracellular domain.[17,120] In vitro, both Dll1 and Dll4 Notch ligands have the capacity to drive T-cell development, although the physiological ligand Dll4 appears to do so more efficiently than Dll1.[121,122] After ligand-receptor binding, Notch1 undergoes ADAM10-mediated cleavage at the S2 extracellular site, followed by gamma secretase-mediated release of the Notch1 intracellular domain (ICN1).[26,123,124] ICN1 acts in the nucleus through a canonical CSL/RBP-Jk and MAML-dependent pathway.[48,125]

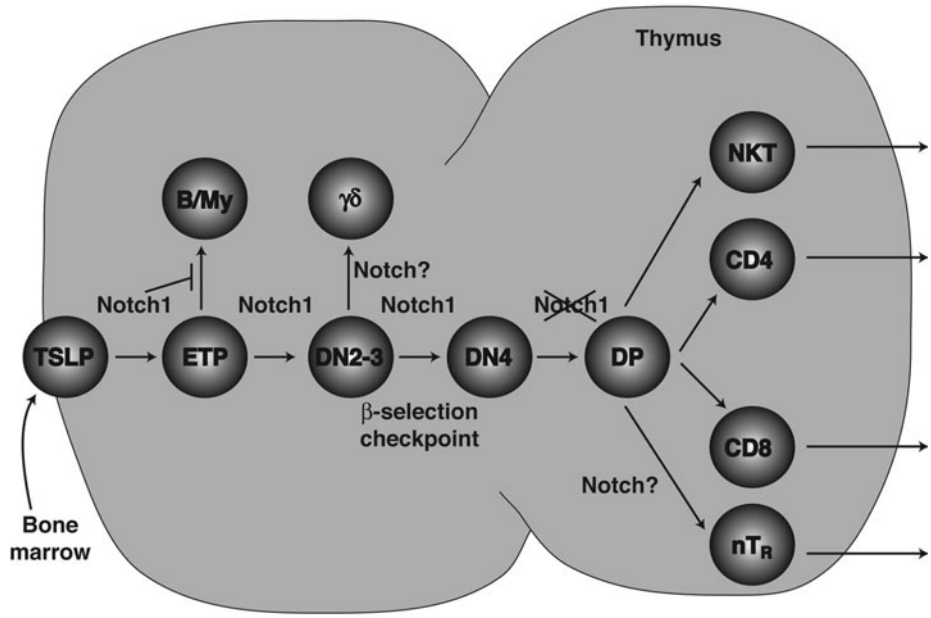


Figure 6. Notch and T-cell development. Notch1 and Dll4 interactions in the thymus are absolutely required during early stages of T-cell development. ETPs, double negative 2 (DN2) and DN3a cells experience a high intensity of Notch signaling. Active Notch signaling during early stages of T-cell development leads to inhibition of both B-cell and myeloid lineages. At the β-selection checkpoint, Notch signaling is rapidly turned off as a consequence of preTCR signaling. Hence, double positive (DP) T-cells experience a very low intensity of Notch signaling. TSLP: Thymus-seeding Lymphoid Progenitor; ETP early T lineage progenitor; DN: $CD4^-CD8^-$ double negative; DP: $CD4^+CD8^+$ double positive; NKT: NK T-cell; nTr: natural regulatory T-cell.

Interestingly, despite more than a decade of research, the nature of the key target genes mediating Notch1's effects in the thymus remains to be clarified. Notch is first required upstream of early T lineage progenitors (ETP), likely immediately after entry of the rare thymus seeding cells.[60,61] However, it is possible that Notch may already operate at prethymic sites, at least in certain circumstances. This has been suggested to happen in fetal T lineage development and demonstrated in the spleen and lymph nodes at early time points after bone marrow transplantation.[126,127]

Notch signaling is most intense in the ETP, double negative 2 (DN2) and DN3a stages of early T-cell development.[60,61,128,129] During these stages, Notch is important to progressively restrict developing progenitors to the T-cell lineage.[130-132] At least in mice, the γδ T-cell lineage appears to be less stringently dependent on Notch signaling than αβ lineage cells, although the situation may be different in human thymopoiesis.[133-135] Notch provides survival signals at least in part due to regulation of cellular metabolism by the PI3K/Akt signaling pathway.[136] Notch has also been reported to regulate c-myc expression and whether this is functionally important in early thymocytes remains to be fully explored.[137] In developing human thymocytes, Notch was also found to bind to and activate the *Il7r* promoter.[138] At the β-selection checkpoint, Notch signaling is rapidly turned off via antagonism of E2A-mediated *Notch1* transcription by Id3, downstream of pre-T-cell receptor signals.[139] As a result, the intensity of Notch signaling in $CD4^+CD8^+$ double positive (DP) thymocytes is very low. Therefore, loss-of-function Notch models do not reveal a physiological requirement for Notch signaling at this stage, even when forced induction of Notch signaling exerts significant effects.[140-143] This active downregulation of Notch signaling may be physiologically important to avoid interference with positive and negative selection in DP thymocytes and their progeny. In addition, unregulated forced expression of Notch signaling can cause T-ALL, highlighting the need for careful regulation of Notch signaling intensity during T-cell development.[7,8]

Altogether, Notch plays an essential role at multiple, successive stages of T-cell development. Interestingly, emerging evidence indicates that Notch is also a major context-dependent regulator of mature T-cell biology,[65,66,144] highlighting the recurrent use of a single regulatory pathway to achieve different effects in the T-cell lineage. Despite all the information gathered on Notch in T-cells, much remains to be learned about the crosstalk between Notch and other pathways, as well as about the downstream molecules that mediate Notch's effects in specific contexts.

OTHER FUNCTIONS OF NOTCH IN HEMATOPOIESIS

In addition to its effects on HSC emergence and its effects in the T-cell lineage, Notch has been reported to regulate marginal zone B-cell development in the spleen, megakaryocyte development, dendritic cell development, as well as peripheral B-cell and T-cell differentiation and function.[14,59,62-66] Due to space limitations, all these effects cannot be reviewed in detail here. However, the regulation of marginal zone B-cell development by Notch deserves mention, since it was shown to be specifically mediated by the Notch2 receptor, the Dll1 Notch ligand and Maml1, a transcriptional coactivator of the Mastermind-like family (Maml1-3).[14,15,145] This indicates a high degree of specificity involving only certain members of the pathway at multiple levels. In future studies, it will be interesting to systematically evaluate if other functions of Notch signaling in the hematopoietic system also rely nonredundantly on specific elements of the pathway.

This may also uncover situations in which targeting of individual Notch receptors and ligands could provide therapeutic benefits while avoiding the side effects of systemic pan-Notch inhibition.

CONCLUSION

As outlined in this chapter, Notch signaling plays multiple functions in the hematopoietic system. Its effects range from an essential role in supporting the development of the first definitive HSCs during fetal life, all the way to profound effects in multiple hematopoietic lineages and mature cells of the immune system. Progress in the field will continue to bring fundamental information about the molecular regulation of hematopoietic development. In addition, practical insights will be gained to use or to target Notch signaling in various applications. For example, exposure to recombinant Notch ligands is being studied as a way to expand hematopoietic progenitors in culture. Another application could be the directed differentiation of embryonic stem cells or induced pluripotent stem cells into definitive HSCs, since efficient protocols to achieve this goal are likely to include the signals that operate normally in vivo during HSC emergence. Furthermore, therapeutic inhibition of Notch signaling could be useful to treat Notch-driven hematopoietic malignancies and to achieve beneficial immunomodulation in specific immune disorders.

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CHAPTER 7

NOTCH SIGNALING IN LUNG DEVELOPMENT AND DISEASE

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Abstract: Notch signaling plays an essential role in development and homeostasis of multiple organs including the lung. Dysregulation of Notch signaling has been implicated in various lung diseases including lung cancer. Here we review functions of Notch signaling in coordinating events during lung development, such as early proximodistal fate generation and branching, airway epithelial cell fate specification, alveogenesis and pulmonary vascular development. We also discuss roles of Notch in chronic obstructive pulmonary disease, progressive pulmonary fibrosis, pulmonary arterial hypertension, asthma and lung cancer.

INTRODUCTION

The Notch signaling pathway is ideally suited to precisely regulate cell-cell communication during development of complex tissues like the lung, where multiple cell types must control each others' survival, proliferation, differentiation and patterning. It has long been known that the lung is amongst the richest source of Notch ligand and receptor mRNA. As more information has come to light about how Notch activation is regulated in vivo, it has become increasingly clear that this system is very tightly controlled through cell autonomous and cell non-autonomous mechanisms. For example, the Delta/Dll-family ligands can be cell non-autonomous agonists that efficiently activate Notch on the surface of neighboring cells,[1] especially if Notch has been modified by a Fringe glycosyl transferase.[2] Serrate/Jagged family ligands function as antagonists capable of inhibiting the Delta-mediated activation of Notch in this context.[3] In contrast, Serrate/

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Jagged ligands function as Notch agonists in cells that do not express Fringe proteins.[2-4] Finally, Delta/Dll and Serrate/Jagged ligands function cell autonomously to inhibit Notch receptor activation.[1] Thus, activation of a specific Notch receptor in a specific lung cell will depend on whether the cell in question expresses one or other Fringe protein, whether it expresses one or other Notch ligand (to block Notch activation) and to what extent neighboring cells express agonistic ligands in excess of antagonistic ligands.[2] This complexity allows for precise coordination of lung cell type specification as outlined below. Unfortunately, the importance of tight regulation of Notch receptor activation in various lung cell types is illustrated by the many pathological states in the lung associated with inappropriate Notch signaling.

NOTCH PROMOTES PROXIMAL CELL FATES IN EARLY LUNG DEVELOPMENT

The lung is a highly branched organ that develops from definitive endoderm of the embryonic gut (beginning at approximately four weeks in humans and embryonic day 9.5 (E9.5) in mice). Epithelial differentiation varies along the proximodistal axis, with the surface epithelium of proximal airways (trachea and bronchi) consisting of basal cells, ciliated cells, goblet cells and neuroendocrine cells. Also found in the proximal region are submucosal glands, which consist of a mixture of mucous and serous cells. Distal to the trachea and bronchi is an extensive network of bronchioles which are lined with ciliated cells and Clara cells and rare populations of neuroendocrine cells. Alveoli are the most distal part of the lungs and are lined with flattened Type I pneumocytes and cuboidal surfactant-producing Type II pneumocytes. While airway epithelia of humans and rodents are similar, there are differences. Notably, in proximal airways of mice there are fewer basal cells, goblet cells and submucosal glands, but a large number of Clara cells. In humans, Clara cells are found only in bronchioles.

Notch signaling pathway genes are expressed in the developing lung as early as bud formation. Within budding epithelium, expression of Notch1, Jagged1 and Jagged2 are restricted to the distal area, while Dll1 expression is found in the proximal region.[5,6] These expression patterns raise the possibility that Notch signaling may control cell fate specification along the proximodistal axis. In experiments on E8.5 murine lung explants, inhibition of Notch signaling with γ-secretase inhibitor (DAPT) causes expansion of Nkx2.1-expressing distal tip progenitors.[5] Older, DAPT-treated, explants (E11.5) display ectopic buds in proximal regions and an increase in the number and size of distal buds. These effects are likely caused by inhibiting Notch1, as antisense oligonucleotides targeting this gene cause increased branching in cultured embryonic lung buds, while antisense oligonucleotides against other Notch receptors do not.[6] Concomitant with an increase in distally-fated cells, DAPT-treated explants show a reduction in SOX2-expressing proximally-fated cells.[5] SOX2 is necessary for the generation and/or maintenance of several proximal cell lineages such as basal and Clara cells.[7,8] A requirement for Notch signaling in promoting a SOX2 proximal fate is also supported by the finding that conditional deletion of *Pofut1*, which codes for an O-fucosyltransferase essential for Notch protein function, strongly reduces SOX2 expression in E18.5 embryos.[9] Activation of Notch signaling at this stage may be driven by FGF10, a regulator of early lung morphogenesis. In E11.5 lung explants, engraftment of an FGF10 bead caused increased Notch1, Jagged1 and Jagged2 expression.[5] Thus, FGF10 may simultaneously promote early proximal

and distal cell fates, with the former being driven by induction of Notch1 signaling. Two complications to this model, however, are the findings that conditional deletion of *Pofut1* or *Rbpjk*, the major transcriptional effector of canonical Notch signaling, does not result in overt alveolar defects.[9] Thus, while Notch signaling promotes early proximal fates marked by SOX2 expression, the physiologic role for Notch in regulating distal progenitor cell identity is less clear.

In addition to regulating early proximodistal cell fate, Notch signaling also regulates later cytodifferentiation of specific lineages. These are generated by distinct stem/progenitor cells found within different segments of the airway. In the trachea and mainstem bronchi, $p63^+$ and cytokeratin $5/14^+$ basal cells self-renew and generate Clara (in the mouse) and ciliated cell lineages.[10,11] In bronchioles, $CCSP^+$ Clara cells self-renew and generate ciliated cells.[12] Deletion of one of the Notch target genes, Hes1, results in a mild reduction of Clara cells in bronchial and bronchiolar epithelia, while disruption of Notch signaling through conditional deletion of *Pofut1* or *Rbpjk* results in a dramatic loss of Clara cells and increased ciliated cell number.[9,13] Despite a reduction in $SOX2^+$ cells in these mutant animals, basal and goblet cell numbers are normal at E18.5. These data suggest that while SOX2 expression, promoted by Notch, may not be necessary to generate basal cells, it may be necessary to promote Clara cell generation and/or maintenance. However, ablation of SOX2 in Clara cells results in a loss of Clara cells and their ciliated progeny,[7] while deletion of *Pofut1* or *Rbpjk* results in a loss of Clara cells and an increase in ciliated cell number.[9] It is, therefore, likely that the effect of Notch on Clara cells is independent of SOX2. Furthermore, since the Clara cell lineage is affected in bronchioles, where there are no basal cells, it is also likely that Notch signaling acts within Clara cells to promote their identity rather than on basal cells to promote Clara cell fate. Consistent with this model, Notch reporter activity, as well as the Notch1 intracellular domain ($N1^{ICD}$) are detected in Clara cells and conditional deletion of *Rbpjk* in $CCSP^+$ cells depletes the Clara cell compartment.[14] In an elegant set of experiments, it was shown that, following injury, Clara cells are generated from a population of cells that initially do not receive a Notch signal, but eventually turn it on prior to expression of CCSP.[14] Thus, Notch signaling may not be necessary for maintenance of Clara cell precursors, but for their differentiation. Further support for this comes from the observation that transgenic misexpression of $N1^{ICD}$ in distal epithelial cells via the SPC promoter results in ectopic expression of the Clara cell marker CCSP.[15] "Salt and pepper" staining of Jagged1 in ciliated cells and $N1^{ICD}$ in Clara cells suggest that lateral signaling from ciliated cells promotes Clara cell identity. Indeed, in the absence of such signaling, cells normally fated to become Clara cells differentiate through a default program into ciliated cells.[9,14]

Another lineage regulated by Notch signaling is the pulmonary neuroendocrine cell (PNEC). Evidence for the role of Notch in regulating PNEC differentiation first came from an elegant study by Ito et al.[13] The Notch target gene Hes1 is expressed in nonneuroendocrine cells, whereas Ash1, a neurogenic basic helix-loop-helix transcription factor, is expressed in neuroendocrine cells. Hes1 deficient mice show increased numbers of $Ash1^+$ PNECs, while Ash1 deficient mice lack PNECs.[13,16] This mutually exclusive relationship results from direct repression of the Ash1 promoter by Hes1 and a Hes1-independent ability of Notch1 to promote Ash1 degradation.[17,18] Notch1 may, therefore, drive Hes1 expression and inhibition of PNEC fate, since Notch1 antisense oligonucleotides promote PNEC differentiation and transgenic expression of $N1^{ICD}$ under control of a neuroendocrine specific promoter inhibits PNEC differentiation.[6,19] This model is further supported by the fact that Dll1 is expressed in neuroendocrine cells, whereas Notch1, 2 and 3 are expressed

in nonneuroendocrine cells.[20,21] Thus, Dll1-mediated Notch activation could well induce Hes1 expression and suppress neuroendocrine differentiation through lateral inhibition. An important caveat to this model, however, is the recent finding that conditional deletion of *Rbpjk* in lung endoderm does not strongly affect Hes1 expression or the number of PNECs.[14] Thus, it's possible that under physiologic conditions, a noncanonical pathway might drive Hes1 expression and inhibition of PNEC fate. Such a mechanism could well employ alternative upstream signaling from FGFR, JAK, or ERK kinases.[22,23]

Pulmonary goblet cell fate is also regulated by Notch. However, the physiologic role for Notch in this context has been difficult to precisely define due to the low number of these cells in murine airway epithelium. In murine tracheal explant studies and in human airway cell cultures, Dll4 increases the number of MUC5AC$^+$ goblet cells.[15] Similarly, expression of N1^{ICD} under control of the SPC promoter increases goblet cell numbers in proximal airways.[15] Conversely, gamma secretase inhibitor treatments block IL-13-induced Muc5AC expression in human airway cell cultures.[15] Thus, high levels of Notch ICD appear to drive goblet cell identity in proximal airways and may also be necessary for IL-13-mediated goblet cell differentiation. However, once again, conditional deletion of *Rbpjk* in the lung endoderm does not affect the number of goblet cells, making it difficult to conclusively establish the physiologic role for Notch signaling in goblet cell differentiation.[9] Studies in the gut suggest that Notch regulation of goblet cell differentiation can be complex, with Notch signaling performing opposite roles towards goblet cell fate in mitotic stem cells and postmitotic differentiated cells.[24-26]

NOTCH COORDINATES ALVEOLAR DEVELOPMENT

Alveolar development in the distal lung occurs through coordinated events in three cellular compartments: epithelium, endothelium and mesenchymal stroma. Notch signaling is known to play important roles in cell fate specification and cell differentiation in the parenchyma and vascular compartments. It is therefore not surprising that Notch may regulate alveolar development by coordinating alveolar epithelial differentiation and capillary formation. As noted above, it is not clear whether Notch signaling plays a direct role in regulating distal progenitor cell fate. Ectopic expression of the Notch3 intracellular domain (N3^{ICD}) in distal lung epithelium causes arrest of alveolar epithelial differentiation, with stalled maturation of Type II pneumocytes and no Type I pneumocyte development observed.[27] Similarly, when constitutively activated N1^{ICD} is expressed in distal lung epithelium, alveolar development is completely abolished.[15] Indeed, distal cysts form and cells within these structures do not express alveolar markers.[15] These data are consistent with explant studies in which E11.5 explants incubated with DAPT show increased branching as well as greater numbers of Nkx2.1 and SPC$^+$ cells.[5] Complicating interpretation of these results, however, are the observations that conditional deletion of *Pofut1* or *Rbpjk* in lung epithelium does not adversely affect distal lung development, including formation of alveolar saccules and differentiation of alveolar epithelial cells.[9,14] Similarly, deletion of *Lunatic Fringe* (*Lfng*), an *N*-acetylglucosaminetransferase that modifies Notch receptors to promote Dll ligand-binding and activation causes only mild defects in alveolar epithelium, namely delayed differentiation of Type I pneumocytes.[21] While *Lfng* knockout mice do show defective alveolar development with failed alveolar septation, these phenotypes likely arise from defective differentiation and mobilization of myofibroblast cells, rather than alveolar epithelial cells.[21] Interestingly, universal deletion

of *Rbpjk* from E14.5 to E18.5 leads to similar defects in myofibroblast differentiation without affecting alveolar epithelial cells.[21] In addition, a similar defect is observed in *Notch2*$^{+/-}$*Notch3*$^{-/-}$ compound mutant mice.[21] Together with the expression pattern of Notch receptors and ligands in the distal lung during cannalicular and saccular stages, these data support a model whereby *Lfng* functions to enhance Notch signaling in myofibroblast precursor cells, thereby coordinating differentiation and mobilization of myofibroblasts required for alveolar septation.[21]

Alveolar development necessarily involves penetration of the growing microvascular network into alveolar walls. This event has to be tightly coordinated with development of alveolar epithelium to ensure lung function at birth. Expression of Notch pathway genes in lung vasculature increases progressively from early to late lung development, suggesting an essential role for Notch signaling in the expanding microvasculature during alveolar development.[20,21,28] This idea is consistent with the known role for Notch signaling in vascular development throughout the body.[29-34] Many Notch ligand and receptor gene knockouts, including Notch1, Notch2, Jagged1 and Dll1 mutants die relatively early in embryonic development, preventing direct analysis of Notch activation in vascular development of the distal lung. Some evidence for the role of Notch signaling in pulmonary vascular development comes from *Foxf1* heterozygous mutants, where *Foxf1* haploinsufficiency disrupts pulmonary expression of Notch2 and its downstream target Hes1. This is associated with abnormal morphogenesis of lung microvasculature and neonatal lethality, although the cell type directly affected by *Foxf1* haploinsufficiency and reduced Notch2 expression remains unclear.[35] Notch3 and Notch4 mutant mice are viable and have at least superficially normal lungs.[36-38] However, expression of constitutively activated N4^{ICD} in vascular endothelium results in lung arteriovenous shunts.[39] As in other contexts, aberrant Notch activation may inhibit vessel sprouting, leading to vessel enlargement at the capillary bed interface and ultimately to arteriovenous shunts.

THE ROLE OF NOTCH IN REPAIR AND DISEASE OF THE ADULT LUNG

Notch signaling regulates development of airway epithelium, mesenchymal stroma and pulmonary vasculature as noted above. Notch also regulates these and other cell types in the adult lung. Minor injuries that require repair occur continuously in mature lungs. Given that Notch activation controls stem cell maintenance and differentiation, cell proliferation and apoptosis, it is not surprising that Notch signaling could be directly involved in the response of lungs to injury. Chronic Obstructive Pulmonary Disease (COPD), often caused by smoking, is associated with down-regulation of Notch pathway genes, including Dll1, Notch3, Hes5, Hey1 and Hey2.[40] Thus, it is speculated that reduced Notch signaling in this context may promote differentiation of airway epithelial progenitors during the repair of lung injury. Further studies on the functional significance of Notch pathway downregulation in COPD should provide insight into its role in disease establishment or progression.

Progressive pulmonary fibrosis is a common and ultimately fatal disease of the lung. It is characterized by fibroblast proliferation, extracellular matrix deposition and chronic remodeling of airways. de novo emergence of myofibroblasts plays a key role in pathogenesis of pulmonary fibrosis. TGF-β and other fibrogenic cytokines are known inducers of myofibroblast differentiation. Recently, Notch1 activation has been shown

to induce myofibroblast differentiation by directly stimulating α-SMA expression[41] and upregulation of Notch1, as well as Jagged1 and Hes1, appears to be downstream of *Found in inflammatory zone 1* (FIZZ1) in myofibroblast induction.[42] It is interesting to note that Lfng-mediated Notch signaling is also required for physiological myofibroblast differentiation during lung alveogenesis, although different Notch ligands and receptors are likely involved.[21]

Pulmonary Arterial Hypertension (PAH) is characterized by a progressive increase in pulmonary vascular resistance leading to right ventricular overload and eventually to right ventricular failure and death. Histopathological features of PAH include thickened vessel wall and luminal occlusion of the small pulmonary arteries and arterioles due to proliferation of vascular Smooth Muscle Cells (vSMC) and endothelial cells. Notch signaling has been shown to regulate differentiation and homeostasis of vSMC. In the developing lung, RBPJκ–mediated Notch signaling is required for recruitment and specification of arterial vascular smooth muscle cells.[14] Recently, Li et al reported that Notch3 signaling promotes pulmonary arterial hypertension.[43] Indeed, human pulmonary hypertension is associated with elevated *NOTCH3* expression in smooth muscle cells of small pulmonary arteries and disease severity correlated with NOTCH3 protein level. Deletion of *Notch3* in mice prevented pulmonary hypertension in response to hypoxic stimulation.[43] Furthermore, pulmonary hypertension can be successfully treated in mice by administration of γ-secretase inhibitor that blocks Notch3 signaling. HES-5 was downstream of Notch3 in this context and controlled proliferation as well as pulmonary vSMC phenotypes.[43]

Notch signaling plays essential roles in development of hematopoietic cells (reviewed in other sections of this book). Many lung disorders, including chronic inflammation and asthma, are mediated by an inappropriate and/or sustained immune response. Notch ligands Dll4 and Jagged1 regulate differentiation and function of T-cells in response to viral or mycobacterial infection in the lung. Dll4 regulates disease pathogenesis during respiratory viral infections by modulating Th2 cytokines to maintain a Th1 environment.[44] During a mycobacterial challenge to the lung, dendritic cells induce differentiation of Th17 cells through a TLR9 effector pathway that upregulates Dll4. Decreased expression of Dll4 in this context led to abrogation of the Th17 phenotype in *Tlr9*$^{-/-}$ mice, with concomitant increase in granuloma size.[45] In an allergic airway condition such as asthma, Dll4 and Jagged1 exerted opposite effects. Dll4 was preferentially expressed by regulatory T-cells to suppress neovasculature remodeling of the airway via proapoptotic Dll4-mediated Notch signaling, therefore alleviating airway hyperresponsiveness in chronic asthma.[46] In contrast, Jagged1 helped initiate lung allergic responsiveness. Indeed, interactions between Notch receptors on CD4^{+} T-cells and Jagged1 on APCs can stimulate IL-4 production and Th2 differentiation, leading to airway hyperresponsiveness and allergic airway inflammation.[47]

NOTCH AND LUNG CANCER

Lung cancers are histologically and molecularly diverse. One sub-group, Small Cell Lung Cancer (SCLC), has neuroendocrine-like molecular features, whereas all other types do not and are referred to collectively as non Small Cell Lung Cancer (NSCLC). SCLCs, which express PNEC markers including Ash1, show no evidence of Notch pathway activation. As discussed above, Ash1 null mice do not have PNECs, as

assessed using canonical neuroendocrine markers.[13,16] Similarly, knockdown of Ash1 in SCLC causes suppression of neuroendocrine marker gene expression, as well as growth arrest and apoptosis.[16,48,49] Interestingly, activated $N1^{ICD}$ or $N2^{ICD}$ can both suppress Ash1 expression in SCLC, an effect reminiscent of Notch-induced suppression of Ash1 in development.[17,18,50] This effect, not surprisingly, is associated with Notch-induced growth arrest of SCLC cells.[46]

The first evidence of an oncogenic role for Notch in lung cancer came from studies on a tumor-associated translocation between chromosome 15 and 19. Overexpression of wild-type NOTCH3, which maps to chromosome 19 near the breakpoint, was observed in this tumor.[51] Interestingly, this NOTCH3 expressing tumor was poorly differentiated and could not be assigned to a particular histological class of lung cancer. Indeed, expression of $N3^{ICD}$ in distal lung epithelium of SPC-$N3^{ICD}$ transgenic mice, causes a dramatic block in differentiation of alveolar cell types.[27] Elevated wild-type NOTCH3 expression has subsequently been observed in 30-40% of primary human lung tumors, typically at a level of expression and/or activity that allows for histological classification.[52] Notch3 is frequently co-expressed with EGFR in NSCLC. In lung cancer cell lines co-expressing both receptors, suppression of Notch3 activity sensitizes cells to EGFR inhibitors.[52,53] These data suggest the Notch3 and EGFR pathways cooperate to promote tumorigenesis. One potential mechanism for such cooperation may be through suppression of the pro-apoptotic gene, Bim.[54] However, another potential mechanism for cooperation could involve EGFR-induced activation of an IL-6 to carbonic anhydrase IX (CA9) pathway, which promotes survival of cells growing under hypoxic conditions. Lung cancers with mutant EGFR show IL-6/Stat3-induced cell growth.[55,56] In breast cancer cells, IL-6 is also frequently elevated, which can lead to increased expression of Jagged1, Notch3 and CA9; as well as enhanced invasiveness and survival under hypoxic conditions.[57] Since Notch3 induction and signaling are required for the pro-tumorigenic effects of IL-6 in breast cancer cells, it is possible that Notch3 may play a similar role in lung cancer cells.[57]

In addition to NOTCH3, other Notch family members display elevated expression and/or activity in NSCLC.[53] Furthermore, 30% of primary lung cancers show severely reduced or loss of Numb expression. Numb is a conserved inhibitor of Notch signaling and absence of Numb expression inversely correlates with activation of Notch1.[58] 10% of NSCLC samples show gain-of-function mutations in NOTCH1 that affect heterodimerization or PEST domains, as observed in T-cell ALL. Furthermore, in tumors without p53 mutations, the presence of activated NOTCH1 correlates with poor prognosis.[58]

Recent analyses of Notch receptor expression and signaling in lung cancer cell lines have been performed under hypoxic conditions, which may better mimic the environment experienced by lung tumor cells in vivo.[59-61] In one study it was found that Notch1 signaling is higher under hypoxic conditions and that this signaling is necessary for cancer cell survival.[60] In contrast, under normoxic conditions, $N1^{ICD}$ promotes apoptosis.[60] Survival is likely mediated, in part, by $N1^{ICD}$-induced stimulation of IGF1R transcription, which promotes AKT phosphorylation.[61] It is also possible that hypoxia-induced expression of HIF1α might cooperate with $N1^{ICD}$ to promote survival, similar to their cooperative interactions in maintaining hypoxic neuronal and myogenic stem/progenitor cells in an undifferentiated state.[62] Collectively, these studies highlight the importance and complexity of Notch signaling in lung carcinogenesis, with the cellular (SCLC vs NSCLC) and microenvironmental (hypoxia) context profoundly effecting tumor cell response to Notch activation.

CONCLUSION

Over the past decade, emerging evidence has established that Notch signaling regulates lung development as well as pathological conditions of the lung. In the developing lung, Notch signaling promotes early proximal cell fates, regulates later cytodifferentiation of airway epithelium into specific lineages (Clara cells versus ciliated cell; neuroendocrine cells versus nonneuroendocrine cells) and coordinates alveolar development. In pathological conditions such as COPD, fibrosis and lung cancer, Notch signaling may exert its impact by regulating stem cell activity, cell differentiation, cell proliferation and apoptosis, reminiscent of its roles during lung development. For instance, COPD is associated with down-regulation of Notch pathway genes, suggesting a role for Notch during repair of injured lungs. Notch pathway exhibits differential properties of tumor promotion or suppression depending on the type of lung tumor. NSCLC show increased Notch signaling and may actively depend on this pathway for tumor cell survival, whereas Notch signaling is not activated in SCLC and may even inhibit growth of these tumors. Notch genes play critical roles in both normal and cancer stem cells of many tissues. They likely function to control survival, self-renewal and differentiation of lung stem cells as well. Future studies to define the expression and function of Notch ligands, receptors and target genes in specific lung lineages as well as in stem and progenitor cells throughout the pulmonary epithelial stem cell hierarchy should help define precisely how Notch signaling regulates lung development and disease.

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CHAPTER 8

THE ROLE OF NOTCH SIGNALING IN KIDNEY DEVELOPMENT AND DISEASE

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Abstract: The kidney is the body's filter, responsible for the removal of metabolic waste and the excretion or reabsorption of electrolytes to control blood composition and pH balance. The functional unit of this filter is the nephron, whose segmented architecture has been largely conserved in form and function throughout eukaryotic evolution. Not surprisingly, the core developmental pathways that regulate the formation of the nephron have also been conserved. In particular, the Notch signaling pathway functions in both primitive and advanced nephrons to pattern domains required for the kidney's diverse functions. In this chapter, we will discuss the role that Notch plays in directing cell fate decisions during embryonic development of the pronephros and metanephros. We will go on to discuss the later role of Notch signaling as a cyst-suppressor and the consequences of aberrant or absent Notch activity in disease and cancer. The work discussed here highlights the fundamental importance of Notch during development and homeostasis of the kidney and underlies the need for mechanistic understanding of its role towards the treatment of human disease.

INTRODUCTION

At its most basic, a kidney functions as a filter, removing toxic metabolic byproducts while maintaining water and electrolyte homeostasis. Virtually all eukaryotes require this function. As multi-cellular organisms began to circulate nutrients, the kidney evolved as a regulator of the circulatory composition under a wide range of environmental conditions and in response to fluctuating nutrient intake. As more complex species emerged, the kidney and its filtration capabilities kept pace. Though the basic functional "kidney" unit

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ranges from a single 'H' cell in *c. elegans* to a segmented mammalian nephron composed of several tubular compartments each containing unique epithelial cell types, these organs share structural similarities and deploy conserved molecular mechanisms to govern their development. Not surprisingly, the evolutionarily conserved Notch signaling pathway regulates multiple aspects of kidney development across phyla.

The vertebrate kidney is derived from the intermediate mesoderm (IM), which is located between the somites and the lateral plate mesoderm of the developing embryo (Fig. 1A).[1] In mammals the IM differentiates sequentially from anterior to posterior into three distinct evolutionary forms of the kidney: the pronephros, the mesonephros and the metanephros. Lower vertebrates, like fish and amphibians, form only a pronephros and mesonephros, the later being the permanent kidney. In higher organisms the pronephros is a transient and vestigial structure, the mesonephros degenerates in females and is

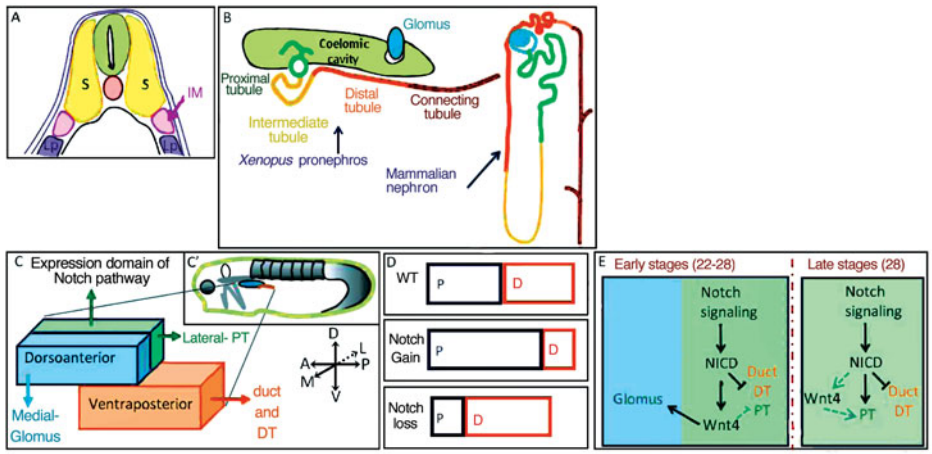


Figure 1. Notch signaling promotes proximal fate differentiation within the pronephros. A) A schematic representation of a transverse section of a Stage 28 *Xenopus* embryo, at approximately the level of somites 3-4. By the end of gastrulation, the intermediate mesoderm (IM, pink region and pink arrow) is located between the Somites (S, yellow) and the Lateral plate mesoderm (Lp, purple). B) The *Xenopus* pronephros and the mammalian metanephric nephrons have similar segments based on gene expression profiles. Each color represents an analogous nephron segment based on similar gene expression profiles. C) Model of pronephric segmentation within the kidney field. A Schematic representation of the pronephros in Stage 26 *Xenopus* embryo (C′). The pronephros can be divided into the dorsoanterior segment (blue) and the ventroposterior segment (orange). The dorsoanterior region can be further divided into a medial region which differentiates mainly to Glomus (light blue) and lateral which differentiates to proximal tubules (PT) (green). Ventroposterior region (orange) will give rise to the duct and distal tubules (DT). Around Stage 22 in the *Xenopus* embryo, hallmarks Notch signaling begin to be expressed in the lateral segment of the dorsoanterior region. On the right, orientation of the 3D axis. A- Anterior, P-Posterior, D-Dorsal, V-Ventral, L-Lateral, M-Medial. D) A representation of how Notch signaling alters the size of the Proximal and Distal regions of the pronephros. Compared to the wild-type (WT) increased Notch signaling (gain) enlarges the proximal region, whereas loss of Notch signaling results in loss of proximal cell fates (blue- proximal, P, orange-distal, (D). E) The early and late stages of proximal pronephros differentiation are regulated by Notch signaling. At early stages (Stages 22-28), Notch signaling induces Wnt4 expression. Wnt4 is secreted to the medial segment permitting glomus differentiation. Notch signaling inhibits the duct, distal tubules and proximal tubules in the lateral segment of the dorsoanterior region. At later stages (around Stage 28), Notch signaling promotes proximal tubule differentiation in the lateral region of the dorsoanterior pronephros and inhibits the differentiation of the distal segment at this region (for more details see the text).

incorporated into the developing epididymis in the male reproductive system. The metanephros is the definitive kidney. The pronephros appears to arise de novo from cells in the anterior IM, whereas formation of the mesonephros and metanephros within the IM relies on mutual induction between an epithelial tube known as the nephric duct (ND) and adjacent mesenchyme.[1,2]

Though different in their overall complexity, each of these kidney structures can be viewed as containing the same functional unit, the nephron. The zebrafish pronephros contains a single nephron, whereas the mouse kidney has more than ten thousand and the human more than one million. Nonetheless, the structure and organization of these nephrons is highly conserved [for review see refs. 3-6 (Fig. 1B)]. All are composed of a glomerulus that functions as the blood filtration apparatus, followed by a tubular structure that secretes and reabsorbs solutes. The tubule connects to a collecting duct which makes the final adjustments to composition of the filtrate before urine excretion (Fig. 1B). Each of these parts is populated by highly specialized cells that are organized in contiguous communities along the proximal-distal axis (glomerulus to the collecting duct). Each cell type is responsible for a particular functional aspect. In the mammalian kidney, for instance, there are more than twenty distinct epithelial cell types in each nephron.

This chapter will focus on the known roles for Notch signaling in formation of the kidney. In the first part of this chapter, we will discuss how Notch governs development of the pronephros, an ancestral form of the earliest vertebrate kidney. Then, focusing on the development of the metanephros in mammals, we will discuss the roles Notch plays in nephron morphogenesis and in cell fate specification within the collecting ducts. The chapter will conclude with a discussion of kidney diseases that arise as a consequence of aberrant or attenuated Notch signaling.

NOTCH SIGNALING IN PRONEPHROS DEVELOPMENT

The expression and function of many genes involved in metanephric development are conserved in the pronephros, including several Notch pathway components (for review see refs. 4-9). Moreover, studies in Xenopus and zebrafish have found that the nephrons in the pronephric kidney have a segmental organization reminiscent of the mammalian metanephric nephron (Fig. 1B).[3-6] The morphological, physiological and molecular similarities between complex and ancestral kidneys indicates that insights from studies in organisms with pronephric kidneys, that are more amenable to genetic modifications, will be relevant to understanding early events governing nephrogenesis across species.

The first markers of the kidney are expressed in a specific position along the anterior-posterior axis when the IM starts to differentiate. Several studies have shown that signals secreted from the axial and paraxial mesoderm are involved in the induction of kidney morphogenetic field within the IM.[1,10-15] Several hours later, as differentiation proceeds within the kidney field, the tubules and collecting duct are specified. In *Xenopus laevis,* the dorsoanterior region will differentiate into proximal tubules (PT) and glomus while the ventroposterior region will differentiate to distal tubules and duct. The medial dorsoanterior region of the kidney field is subsequently patterned into the glomus and nephrostomes and the lateral region gives rise to PT (Figs. 1C,C′).[5,16] The transcription factors Hox and Caudal (cdx) and retinoic acid signaling play a key role in both positioning the pronephros along the anterior-posterior axis of IM, as well as in establishing proximal-distal segment differentiation within the pronephros.[15,17]

Work in *Xenopus laevis* has interrogated the role of Notch signaling in patterning the pronephros.[18,19] Here, Notch pathway components including the receptor Notch1 and the ligands Serrate1, Serrate2 and Delta1 have a dynamic, biphasic expression pattern. In the early phase (Stages 21-32), expression is confined to the lateral segment of the dorsoanterior region of the pronephric anlagen (Fig. 1C). Later (Stages 33-34), expression of Notch1 and Serrate1 can be detected in the forming PT. Other genes which modify Notch signaling, such as fringe proteins, are specifically expressed only during the early phase.[20] In agreement with this observation, in the zebrafish embryo orthologs of the Notch ligands (DeltaC, Jagged1b and Jagged2a) were also shown to be expressed in the proximal region of the pronephros.[21,22]

Activation of Notch signaling using inducible, constitutively active Notch1 ICD (NICD1) or the transcription factor Suppressor of Hairless (Su(H)) leads to repression of pronephric duct and distal tubule markers c-Ret, Lim-1 and Evi1 and an increase in proximal markers Pax-2 and WT-1. Conversely, inhibition of endogenous Notch signaling with inducible dominant negative Su(H) leads to an increase in the expression of duct and distal tubule markers.[18,23] Hence, it appears that Notch signaling functions to promote formation of the proximal compartment and may play a role in repressing duct and distal tubule differentiation in the dorsoanterior region of the pronephros (Fig. 1D).

Recent studies have shown that Notch regulates the differentiation of the proximal compartment in a temporally distinct manner (Fig. 1E).[19,20] Mis-activation of Notch during the early phase causes the entire anlagen to acquire glomus fate at the expense of PT, while later activation had no effect on the glomus domain. In some of these embryos the PT domain was expanded. These data suggest that Notch signaling is directing glomus formation during the early phase and is required later for PT differentiation.

These functions of Notch are likely mediated, at least in part, through the Notch target gene Hairy-related transcription factor (xHRT1, also known as Hey1) which is expressed in the proximal domain of the pronephros (dorsoanterior region). When xHRT1 translation was inhibited, a reduction in the expression of proximal markers was observed.[24]. Conversely, when xHRT1 was over-expressed, distal tubule and duct cell fates were inhibited, similar to what was seen in Notch gain of function mutants. Corresponding increases in PT and glomus formation, however, were not observed. Moreover, in embryos where Notch has been inhibited, xHRT1 did not restore the expression of PT markers, suggesting that only part of the effect executed by Notch is mediated by xHRT1.[19,25] This is supported by data in the mouse where Hey1 mutant mice display no overt kidney phenotypes.[24] Together, these data indicate that both in the pronephros and in the metanephros, Notch function is likely mediated by a constellation of target genes, rather than by a single key player.

Studies in the pronephros have shown that Notch regulates glomus-PT patterning (medio-lateral patterning) in a Wnt4 dependent manner.[20] When Wnt4 was over-expressed an expanded glomus domain formed at the expense of PT. Inhibition of Wnt4 translation with morpholinos abrogated the ectopic glomus development in embryos with constitutive Notch activation, indicating that Wnt4 is necessary downstream of Notch to mediate its function. This study went on to investigate the genetic hierarchy of Notch, Wnt4 and HRT1 in patterning the proximal region of the pronephros. Activation of NICD1 or inducible HRT1 leads to ectopic expression of Wnt4, while suppression of Notch signaling or inhibition of HRT1 results in reduction of Wnt4 expression. Moreover, over-expression of NICD1 results in ectopic HRT1 expression while in embryos where Wnt4 was over-expressed, no significant change in HRT1 expression was detected.[19,20] These experiments suggest that HRT1 is regulated by Notch signaling and is required

upstream of Wnt4. This network appears to be a key regulator of proximal fate specification in the pronephros. Whether these interactions are direct or indirect is yet to be determined

The role of Notch signaling in patterning the proximal segment of the pronephros is further supported by studies in which radical fringe (rFng) is over-expressed in the developing pronephros.[20] Fringe proteins are known to modulate the activation strength between ligand/receptor pairs (for review see ref. 26). In this setting, inhibition of rFng had no effect on the development of pronephric domains. However, rFng over-expression seems to overcome the temporal effects of Notch and results in duplication of the entire proximal domain (both glomus and PT) at the expense of the distal domain. The differing results between mis-activation of NICD1 vs rFng suggest that Notch independent pathways may play a role in the differentiation of the proximal pronephric domain.

Since the location and level of Notch signaling dictates the fate of cells within the pronephros it is of importance to identify the regulators of Notch receptor and ligand expression. In this regard, zebrafish embryos deficient in retinoic acid synthesis have abrogated DeltaC and Jagged2 expression in the proximal segment of the pronephros and subsequently an expansion of the distal identity. In further support of retinoic acid playing a role in regulating expression of Notch pathway components, exogenous retinoic acid expands the region of DeltaC and Jagged2 expression.[17]

NOTCH SIGNALING IN METANEPHROS DEVELOPMENT

An Overview of Metanephros Development

Formation of nephrons in the mammalian metanephros follows a highly stereotypical, evolutionarily conserved pattern (for review see refs. 4, 9, 27). The metanephros arises at the level of the hind limb in the far posterior IM at the caudal end of the ND. This occurs through reciprocal tissue interactions between the ureteric bud (UB), emerging from the ND and the adjacent metanephric mesenchyme (MM). At embryonic day 10.5 (E10.5) in the mouse, signals from the MM induce the outgrowth of the UB from the ND. As the UB epithelium invades and forms its initial branch, a subset of cells within the MM surround the UB to form a cap structure (Figs. 2A,B). This contains the progenitors for all nephron epithelia while stromal precursors within the MM segregate outside the cap mesenchyme and gives rise to the interstitium.[28-31] The MM also contains vascular endothelial cell precursors. UB invasion and MM capping initiates mesenchymal-to-epithelial (MET) transition of some MM cells at the UB tips. Those that are not directed to undergo MET remain in the progenitor pool, some of which are capable of self-renewal and will eventually be induced by subsequently formed branch tips (Fig. 2A).[28,30] In turn, the MM promotes the growth and branching of the UB, which will give rise to the collecting duct. This reciprocal process of UB branching and induction of the mesenchyme is repeated throughout kidney development and ends before or shortly after birth when a species appropriate number of nephrons had formed. At each branch tip, groups of induced MM cells can be molecularly identified as the pretubular aggregate (PTA) and will each give rise to one nephron (summarized in Fig. 2A). The PTA quickly progresses to form the renal vesicle (RV), a morphologically distinct structure that has a primitive lumen and some epithelial characteristics. Importantly, asymmetric and polarized gene expression is observed in the RV, suggestive that the process of commitment to the different cell types of the nephron begins early in nephrogenesis, with distal cells differentiating first.[32]

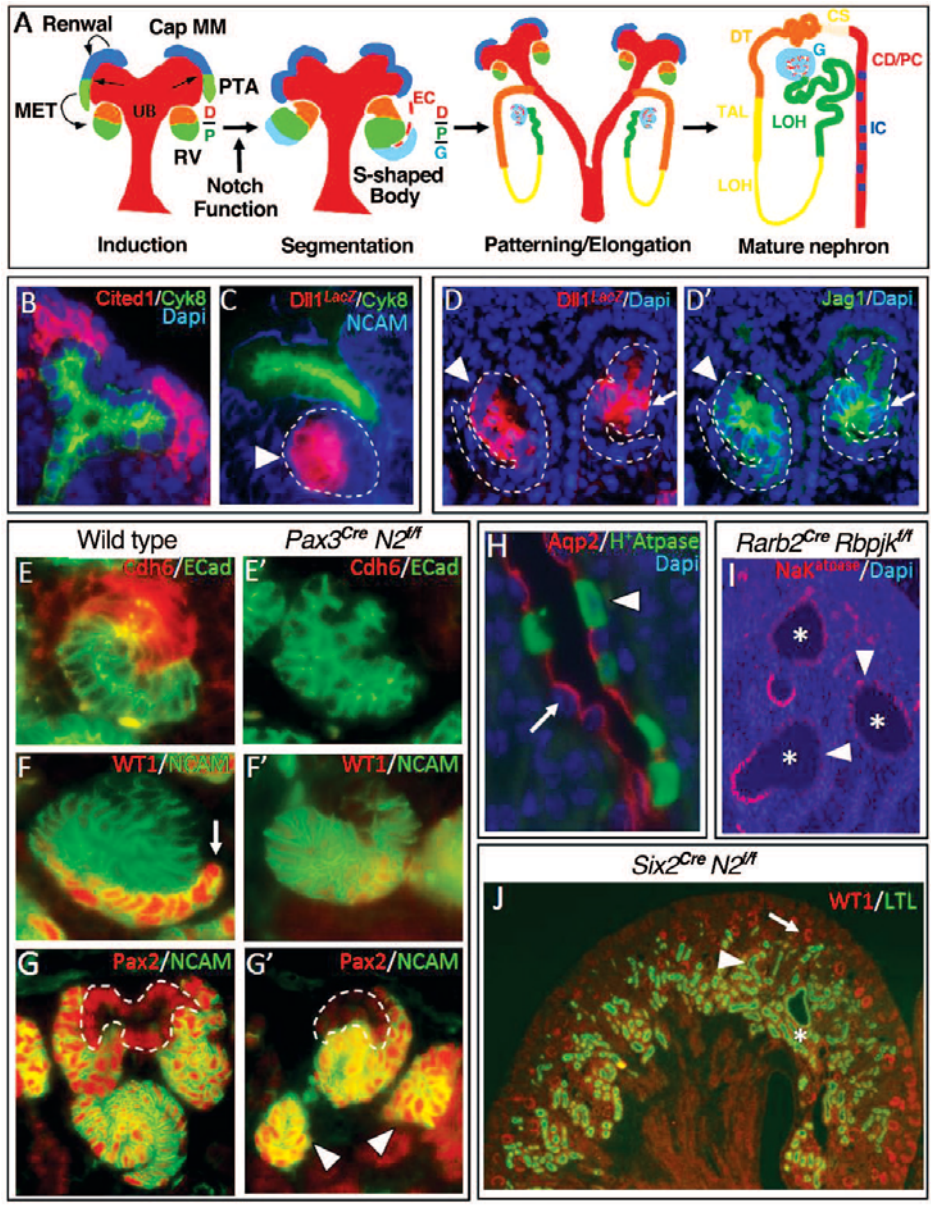


Figure 2. Notch signaling in the metanephric kidney. A) Schematic representation of nephron induction and maturation in the mammalian kidney. **Induction**—Signals from the ureteric bud (UB) induce cells in the cap metanephric mesenchyme (cap MM) to undergo mesenchymal-to-epithelial transition (MET). Induced cells are molecularly identifiable as the pretubular aggregate (PTA), which progresses to form the renal vesicle (RV), an immature epithelium divided into proximal (P) and distal (D) domains. It is during this transition that Notch function is required. The distal RV fuses to the UB, forming the connection between the nephron and collecting system. **Segmentation**—The RV is further divided as it elongates and forms the S-shaped body. A distinct glomerular domain (G) is present. Endothelial cells (EC) invade the proximal cleft, where they will vascularize the glomerulus. Figure legend continued on opposite page.

Figure 2. Figure legend continued from previous page. **Patterning/elongation**—Glomerular, proximal and distal domains are further subdivided and elongate. Terminal differentiation of cell types within segments occurs. New nephrons are continually induced at branching UB tips. **Mature nephron**—The mature nephron can be generally divided (from P to D) into the glomerulus (G), proximal tubule (PT), loop of Henle (LOH), thick ascending limb (TAL), distal tubule (DT) and connecting segment (CS), all of which are derived from the cap MM. The collecting duct is derived from the UB and is made up of interspersed principal cells (PC) and intercalated cells (IC). B-D′-Representative images of early inductive events. B) Cytokeratin 8 (Cyk8) marks the UB and Cited1 marks the cap MM. C, D-D′) Dll1 (C,D arrowhead) and Jag1 (D′ arrowhead) expression is confined to the distal domain of the RV. As the S-shaped body emerges, Dll1 and Jag1 protein are segregated to the middle domain. E-G′) Patterning of S-shaped bodies in wild-type and Notch2 mutant kidneys. Pax3^{Cre} deletes Notch2 in the MM just prior to UB invasion and results in impaired S-shaped body morphogenesis. In mutants, the truncated structures that form during RV to S-shaped conversion contain a distal domain marked by E-Cadherin (ECad), but lack the putative PT cells expressing Cadherin-6 (Cdh6, E,E′) and the glomerular crescent, marked by Wilms' Tumor 1 (WT1, F,F′). Truncated S-shaped bodies do fuse their distal domain (marked by Pax2) to the UB (dashed outlines), but fail to delineate a proximal segment (arrowheads, G,G′). H) Representative distribution of Aquaporin 2 (Aqp2, arrow) expressing principal cells and H^+Atpase (arrowhead) positive intercalated cells in the mature collecting duct. I,J—Delayed inactivation of RbpJκ (I) or Notch2 (J) using alternative Cre lines allows glomeruli (WT1, arrow) and PT (LTL, arrowhead) to form but causes cystic kidney disease (asterisks), demonstrating a later function for Notch signaling in the nephron. The frequency of these cysts is higher in the RbpJκ mutants, indicating an additive function for Notch1 in this process. Down-regulation of Na^+Katpase in cysts indicate loss of epithelial integrity (I, arrowhead).

As these early inductive events generate proximal cell populations, the S-shaped body forms. It is here that segmental identities appear to be set, with distinct gene expression delineating putative glomerular, proximal and distal domains. The distal RV attaches to the UB-derived collecting duct, forming the patent connection that allows the filtrate to exit the kidney. The S-shaped body elongates and directs differentiation of specialized epithelial cells along the proximal-distal axis. The end result of this process is the nephron, composed of more than twenty different cell types divided into functionally distinct domains. Notch signaling plays a central role early in this segmentation process.

Notch Signaling in the Developing Mammalian Nephron

Early evidence of the importance of Notch signaling during development of the metanephros came in humans with the correlation between mutations in Jagged1 (Jag1) and Alagille syndrome (ALGS1).[33,34] ALGS is a congenital, heritable disease which affects multiple organ systems; including the kidney (renal pathologies associated with ALGS will be addressed later in this chapter). Later, mutations in Notch2 were also linked to human cases of Alagille (ALGS2).[35] Importantly it was shown that all ALGS mutations were autosomal haploinsufficent mutations, meaning that one mutant allele was enough to cause disease. This phenomenon was also observed, serendipitously, in the mouse. Here, germ-line deletion of both copies of *Notch2*, through replacement of its ICD with β-Galactosidase, is lethal at mid-gestation due to vascular defects, at the onset of kidney development.[36] Another strategy, however, targeted a different region of the *Notch2* coding sequence that resulted in production of semi-functional splice variants with enough activity to bypass early lethality.[37] Most homozygous mice carrying this hypomorphic allele die within 24 hours of birth with a 50% reduction in kidney size and impaired glomerulogenesis. Importantly, Jag1/Notch2 compound heterozygous mouse mutants recapitulated the spectrum of disease in ALGS patients, including in the kidney.[38] The kidney defects in these mice are not well characterized, but hypoplastic

kidneys (suggesting reduced nephron number) and ruptured glomerular capillaries are evident. These initial discoveries demonstrated that reduced levels of Notch signaling caused pathological changes in nephrogenesis and led to a more detailed investigation of its role in the developing mouse kidney.

A number of Notch pathway components are expressed in the early nephron. These include receptors:Notch1,2,3, ligands: Jag1 and Delta1 (Dll1), known target genes: Hey1, HeyL and modifiers of Notch receptor/ligand affinity, such as Lunatic Fringe.[39-41] Importantly, Dll1 and Jag1 expression is polarized toward the distal end of the RV suggesting that Notch signaling may function in early nephron pattering events (Fig. 2B).[40,42] Later, Dll1, Jag1 and activated Notch1 protein are segregated to the middle of the S-shaped body (Figs. 2C,D,D′).[32,42] Notch2 protein is expressed throughout the RV and S-shaped body, but its activation domain is unclear.[43] The centralized localization of ligands (Figs. 2D,D′) and canonical targets Hes1 and Hes5 suggests that signaling is localized.[44] Though this domain is thought to contain precursors for the PT, Loop of Henle and ascending limb, definitive fate mapping studies have not been preformed. To understand the role of Notch signaling in this process, two independent laboratories performed loss-of-function experiments in the mouse specifically focused on the kidney. These studies relied on disrupting the catalytic component of Notch activation, the γ-secretase complex, either by treating cultured embryonic kidneys with DAPT, a small molecule inhibitor of γ-secretase[45] or through genetic removal of presenilins, which help form the active subunit of the complex.[46] Both studies demonstrated that Notch signaling was not a major regulator of UB branching or the early inductive phase of MET, but that removing γ-secretase activity resulted in the formation of truncated, nonfunctional nephrons. Specifically, nephrons in these mice lacked the most proximal domains, including the glomeruli and the PT. The flexibility of ex vivo drug administration in the DAPT study allowed the investigators to map the temporal requirements for Notch in proximal nephron formation.[45] These studies yielded two important findings. First, Notch signaling was only required for the specification of proximal structures in the early nephron; cells that had already acquired proximal identity continued to differentiate even when drug was added. Second, when drug was removed and the kidneys were allowed to recover, there was a strong correlation between the length of exposure to drug and the types of cells that formed upon its removal. After just 48 hours in DAPT, kidneys lost the ability to form glomerular podocytes; however, PT and distal tubules recovered. After 72 hours in DAPT, no new PT formed and after 96 hours nephrogenesis did not recover. Together, these results show that γ-secretase activity is required in a very narrow developmental window (likely during conversion of the RV to the S-shaped body) and that cell fate decisions are differentially sensitive to Notch pathway inhibition; the more proximal, the more sensitive.

These studies however, had limitations. Most notably, disruption of γ-secretase is a promiscuous method of inhibiting Notch activation that does not discriminate between different Notch receptors or ligands. Furthermore, Notch receptors are not the only substrates cleaved by γ-secretase. To more precisely define the logic of Notch signaling during nephrogenesis, Cheng et al adopted an approach to specifically remove Notch1 and Notch2 from renal progenitors just prior to UB invasion.[42] Strikingly, signaling through Notch2 accounted for all of the activity needed to make proximal nephron structures. Podocytes and PT formed in the absence of Notch1 and Notch1 activity could not rescue loss of Notch2. This is despite the fact that active (cleaved) Notch1 is clearly present in the developing S-shaped body. Furthermore, in vivo mosaic analysis, demonstrated that Notch1-null cells can contribute to all nephron lineages in the kidney. Together, these

experiments demonstrated that Notch2 is absolutely required for specification of the proximal nephron and that Notch1 was largely dispensable.

Detailed analysis of Notch2-null kidneys offered further clues as to how and when its activity is important for nephron segmentation. Notch2 null cells properly respond to inductive signals and form RVs as they exit the MM, but conversion to a polarized S-shaped body is impaired (Figs. 2E-G′). The truncated structures that do form express E-Cadherin and Pax2 (early distal markers, Figure 2E′,G′) and properly connect to the CD. They lack, however, markers of the proximal domain, including Cadherin-6 (early PT, Figs. 2E,E′) and WT1 (immature podocytes, Figs. 2F,F′). In mutant S-shaped bodies, Jag1 is expressed, delineating the area where Notch signaling would be active. These cells are at a proliferative disadvantage compared to controls.[42] This may mean that the primary role of Notch activity in the S-shaped body is to promote proliferation of the proximal domain, allowing other pathways to provide instructive, fate-determining signals.

In an effort to understand why Notch2 was dominant in this setting, two reasonable explanations were tested. One explanation could be that Notch2 works through a noncanonical (Rbpjκ independent) pathway in the early nephron. When Rbpjκ was removed from renal progenitors, thereby abolishing all canonical Notch signaling, the phenotype was identical to loss of Notch2. It is also possible that even though Notch1 is active in the S-shaped body, its ICD is incapable of activating the targets necessary to specify proximal fate. On the contrary, ectopic expression of NICD1 in the MM drives all cells to proximal fates at the expense of all other identities, reminiscent of what was seen in the frog and discussed earlier in the chapter. Importantly, this effect is not dependent on endogenous Notch2 activity.[42] More recently, it has been shown that Notch1 makes a small contribution to nephrogenesis.[43] When Notch2 deletion is delayed so that it occurs in the PTA just prior to RV formation, the result is an intermediate phenotype; some nephrons do form, but many kidneys have significantly fewer nephrons. In this sensitized setting, removing alleles of Notch1 has a progressive phenotype, with double nulls having lethal kidney defects. This indicates that though Notch2 is the dominant paralog in the developing nephron, Notch1 can act synergistically to promote proximal fates.

The mechanistic basis for Notch activity during the early stages of nephrogenesis is unclear. How is Notch2 working to direct proximal morphogenesis? What happens to the cells that would have comprised the proximal nephron? For instance, it is possible that the default fate of cells in the early stages of MET is distal. In this case, Notch2 would be acting as a restrictive signal, creating a proximal boundary by blocking the distal fate and allowing parallel, instructive signals to direct fate decisions. As discussed above however, Notch gain-of-function in renal progenitors converts all cells to proximal fates, suggesting an instructive role for Notch signaling in this process. It is also unclear how the loss of glomerular and PT epithelia is linked. Is Notch somehow defining an early, broad proximal domain that contains common progenitors, or is it responsible for directing a specific cell fate, without which the rest of proximal nephron cannot form? This is complicated by the fact that as of now, we do not know when and where Notch2 is active in the RV and S-shaped body, the fate of cells that experience Notch2 activation, or the fate of cells that should have but have not received a Notch signal.

The other major gap in our understanding of how Notch works during nephron patterning are the identities of direct downstream targets that mediate its critical functions. Evidence in mice strongly suggests that it is not a single factor, but a constellation of targets that are required downstream of Notch in the RV and S-shaped body. Deletion of several individual, canonical Notch targets, including Hes1, Hes5, Hey1, HeyL and

Nrarp has failed to recapitulate the Notch2 null phenotype in the kidney (HB and KS unpublished data.).[24,39] This combinatorial program suggests a complex function for Notch in the RV/S-shaped body and has complicated identification of critical targets.

Notch Signaling in Collecting Duct Development

Two main functions of Notch signaling in the developing collecting system have been identified thus far. One function is revealed in the ability of constitutively active Notch signals to regulate the branching pattern of the UB, which gives rise to the ureter and the collecting duct system. As previously discussed, the MM induces the outgrowth of the UB from a caudal region of the nephric duct at E10.5 in mice and E28 in humans. The main inducer of UB formation and a dominant regulator of the subsequent branching of the UB is glial-cell derived neurotrophic factor (GDNF) which signals to the c-Ret tyrosine kinase and GFR1a receptors expressed in the ureteric bud tip cells (for reviewed in see ref. 47). In the wild-type setting, Ret is initially expressed in all regions of the nephric duct, but it becomes restricted to the UB tip after the initial branching of the UB. Whereas inactivation of GDNF/Ret signaling results in the absence of UB formation, ectopic activation of GDNF/Ret signaling by implantation of GDNF coated beads stimulates supernumerary ureteric tips. Jag1 is normally expressed in the UB tips much like c-Ret.[48] However, continuous expression of Jag1 in the nephric duct and its derivatives disrupts the normal branching of the UB resulting in a range of phenotypes including unilateral aplasia, double ureters and tubular cysts.[48] This result is consistent with the observation that ectopic Jag1 expression in the entire nephric duct and UB (and thus, elevated Notch activity in the collecting system) expands Ret and GFR1α expression beyond the UB tip. Whereas ectopic activation of Notch signaling in the UB alters the branching, there is so far no evidence that endogenous Notch signaling regulates UB branching. Only a slight disruption of UB branching occurs with γ-secretase inhibition in cultured kidneys.[45] And this effect of γ-secretase inhibition could be independent of Notch signaling especially since loss of Notch signaling in the collecting system via inactivation of the E3 ubiquitin ligase Mind-bomb1 (Mib1), a protein whose function is required in ligand expressing cells for the efficient activation of Notch receptor, appears not to alter UB branching.[49,50]

Mib1 inactivation in the collecting system instead reveals a role for Notch signaling in the differentiation of duct cell fates.[50,51] The mature collecting duct consists of functionally distinct cell types required for normal acid-base homeostasis and water and electrolyte balance. These include the more abundant principal cells, which are vasopressin responsive and concentrate urine by absorbing water from the lumen of the nephron and regulate Na^+ and K^+ homeostasis and the α and β intercalated cells, which regulate pH homeostasis by secretion of H^+ or HCO^{3-} into the urine (Figs. 2A,H). Unlike the nephron, which is organized into segments consisting of unique cell types, principal and intercalated cells reside intermingled along the entire collecting system with a gradual increase in the ratio of principal to intercalated cell types toward the medulla. The collecting duct specific inactivation of Mib1 results in an increased number of intercalated cells and a reduced number of principal cells. As a consequence of principal cell deficiency, mice with Mib1-deficient collecting ducts excrete much more urine and fail to concentrate their urine following dehydration, which is reminiscent of nephrogenic diabetes insipidus (NDI) in humans.[50] Although loss of Mib1 in the collecting ducts alters the ratio of principal to intercalated cell populations more severely in the medulla than in the cortex, both cell types still form in all regions of the duct. This may reflect compensation for loss of

Mib1 by up regulation of Mib2.[50] Alternatively Notch signaling may be required only in a subset of bi-potential collecting duct progenitors.

The molecular mechanisms by which Notch signaling regulates principal versus intercalated cell fates remain to be determined, but the development of intermingled cell types as well as the non-uniform expression of Jag1 in the developing collecting duct is suggestive of a lateral inhibition mode of cell fate patterning. In this context, the multi-ciliated versus transporting epithelial cell differentiation in the zebrafish pronephros is an example in which Notch signaling patterns cell fates via lateral inhibition.[52,53] As in the collecting duct, the zebrafish pronephros contains intermingled cell types; loss of Notch signaling results in expansion of the minority cell population, the multi-ciliated cells, at the expense of transporting epithelia. This is likely due to the fact that Notch mutants fail to repress Rfx2, a central transcription factor required for the development of multi-ciliated cell characteristics. In the mammalian collecting ducts, the differentiation of the minority population, the intercalated cell type, requires the forkhead transcription factor Foxi1. *Foxi1*$^{-/-}$ mice fail to acidify the urine due to the absence of intercalated cells as evidenced by the lack mitochondria-rich cells with prominent apical microvilli and the complete absence of proteins necessary for proton and bicarbonate secretion.[54] Although the direct targets of Notch signaling that promote principal cell differentiation remain to be determined, Notch signaling directly or indirectly suppresses Foxi1 expression and Foxi1 directly trans-activates the promoters of intercalated cell specific genes.[50,54-56] In both cases therefore a default fate is repressed by Notch signaling to permit an alternative fate to emerge, a hallmark of lateral inhibition.[57]

NOTCH SIGNALING IN KIDNEY DISEASES AND RENAL EPITHELIAL CANCERS

The functions of Notch signaling in adult renal tissue homeostasis are beginning to be studied and appear to be dependent on the duration of Notch pathway activation. Notch signaling is only transiently activated following acute (brief) renal injuries at the time when renal epithelial regeneration occurs and may be involved in the repair process allowing for recovery of renal function.[58,59] On the other hand, chronic renal injuries result in sustained activation of the Notch signaling pathway and promote glomerulosclerosis and loss of renal function.[60,61] In this regard, the constitutive activation of Notch signaling in mature podocytes, which facilitate glomerular filtration, triggers the down regulation of mature markers of podocytes, increases apoptosis and the reentry of remaining podocytes into the cell cycle resulting in defective glomerular filtration.[60,62] This hyper-activation of Notch signaling has been detected in the podocytes of humans with glomerular diseases.[60,63] Furthermore, in rodent models of glomerular diseases the inhibition of Notch signaling using γ-secretase inhibitors or genetic inactivation of RBP-J in podocytes ameliorates the loss of podocytes and glomerular filtration capability.[60] These observations uncover a critical role for sustained Notch activation in the pathophysiology of glomerular diseases.

Additional observations in humans and mice implicate Notch signaling in the prevention of renal cystic disease and renal epithelial cancers. As stated previously, ALGS patients are known to have mutations in either Jag1 (ALGS1) or Notch2 (ALGS2). Interestingly ALGS2 patients have more severe kidney abnormalities than ALGS1 patients, with all affected individuals in two kindreds manifesting renal abnormalities, including one child with bilateral renal cysts.[35] Evidence for Notch pathway mediated suppression

of renal cysts emerged in mice experiencing delayed conditional inactivation of Notch1, Notch2 or Rbpjκ, which allows for nephron segmentation and PT formation.[43] These PTs later form cysts during the nephron lengthening/growth phase (Figs. 2I,J).[64] The frequency of cysts increased as Notch signals decreased; the combined inactivation of Notch1 and Notch2 in the PT resulted in multiple proximal tubular cysts at birth similar to the frequency seen with Rbpjκ deletion.[64]

The mechanisms driving cyst formation in Notch-deficient PT involves epithelial stratification and increased proliferation of epithelia losing contact with the basement membrane (BM). In a normal kidney, tubules grow in length by epithelial proliferation in which the division plane is oriented along the long axis and perpendicular to the BM such that each cell maintains BM contact in a monolayer. Notch signaling is required for maintaining this plane of division in the renal PT during morphogenesis preventing division from occurring parallel to the BM.[64] This function of Notch signaling is required to develop a consistent PT diameter and to maintain a polarized epithelial monolayer. The Notch-signaling deficient PT epithelia have partially randomized spindle orientation relative to the BM coupled with increased numbers of mitotic events that combine to result in epithelial stratification prior to overt cyst formation. The mitotic spindle orientation is also restricted to the long axis of the nephron during renal epithelial repair following acute injuries in adult mice.[65] Since Notch signaling is reactivated during nephron repair it is possible that Notch signaling may be required in the adult kidney for the prevention of cyst formation during recovery from injury.[58]

The stratification of Notch deficient renal epithelia also results in a low incidence of papillary microadenomas, which may be a precursor state for papillary renal cell carcinoma (PRCC).[64,66] Coincidentally, human Type 1 PRCC is characterized by elevated levels of KyoT3/FHL1B, which can act as an inhibitor of the canonical Notch signaling pathway.[67,68] Other evidence that Notch signaling prevents renal cancers comes from a recent report that Notch1 and Notch4 are expressed in normal human kidneys but greatly reduced in human renal cell carcinomas.[69] These observations lead to the speculation that the canonical Notch signaling pathway actively suppresses papillary renal cell carcinoma in humans.

CONCLUSION

Most of the functions of Notch signaling identified thus far in the kidney reinforce the role of Notch in directing the differentiation of neighboring cells into distinct cell fates. In the pronephros and metanephros Notch activation appears to create a precursor proximal tubule domain at the boundary between distal precursors and the cells that will form the filtration apparatus. Forced activation of Notch drives all the cells within the equivalency group to adopt the boundary proximal tubule cell fate. In the collecting duct where two different cell types are intermingled, Notch appears to function via a lateral inhibition mode in which the intercalated cell type likely activates Notch in its neighbors to suppress intercalated fate and allow for the alternative principal cell fate to manifest. The actions of Notch signaling in preventing renal cysts with stratified epithelia, as well as ectopic Notch mediated UB branching are suggestive of an additional direct role for Notch signaling later, during tubule morphogenesis rather than cell fate differentiation. Evidence in the mouse clearly suggests that Notch signaling is deployed in the adult and plays a role in pathogenic processes, though the scope of renal pathologies associated with

Notch in adult humans is unclear. This is an important question given the fact that Notch receptors and ligands are accessible drug targets and underlies the need to understand the mechanisms of Notch signaling in the kidney.

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CHAPTER 9

NOTCH SIGNALING AND THE DEVELOPING SKELETON

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Abstract: Notch signaling is an important regulator of skeletogenesis at multiple developmental stages. The Notch signaling pathway is involved in the promotion of somite segmentation, patterning and differentiation into sclerotome pre-chondrogenic cells to allow for appropriate axial skeleton development. In addition, studies performed in vitro and in vivo demonstrate that Notch signaling suppresses chondrogenic and osteoblastic differentiation and negatively regulates osteoclast formation and proliferation. Through the use of in vitro and in vivo approaches, Notch signaling has been shown to regulate somitogenesis, chondrogenesis, osteoblastogenesis and osteoclastogenesis that ultimately affect skeletogenesis. Dysregulation of Notch signaling results in congenital skeletal malformations that could reveal therapeutic potential.

INTRODUCTION

Skeletogenesis is a complex, multi-step and highly regulated process by which early mesenchymal condensations differentiate to form the skeletal elements in the body. The axial skeleton is primarily formed through somite differentiation into cartilage progenitor sclerotome cells, while the appendicular skeleton of the limbs is formed through chondrogenic aggregates that transition into bone during a process termed endochondral ossification.[1] Most flat bones of the body, including the developing skull bones, form through intramembranous ossification, whereby mesenchymal condensations differentiate directly into bones without a cartilage intermediate.[2] During embryogenesis, there is a tight balance of cartilage forming cells (chondrocytes), bone forming cells (osteoblasts) and bone reabsorbing cells (osteoclasts), to allow for normal patterning and cell type

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contributions to skeletal development. Throughout life, homeostatic mechanisms in the skeleton control bone density, maintenance and cartilage integrity. Dysregulation of these processes can lead to skeletal diseases such as osteoporosis, osteopetrosis, osteoarthritis and osteosarcoma.

The canonical Notch signaling pathway, implicated in skeletal development and disease, consists of five ligands (Delta ligand1, 3, 4, Jagged1, 2) that interact with four Notch receptors (Notch1-4). Upon ligand binding, the Notch receptor is cleaved, first by tumor necrosis factor α conversion enzyme (TACE) and then subsequently by the gamma secretase complex that consists of Presenilin1 and 2 in mammals. Upon receptor cleavage, the Notch intracellular domain (NICD) translocates to the nucleus where it binds to the transcription factor recombination signal binding protein for immunoglobulin kappa J (RBP-J, also termed RBPJK, CBF1, RBPSUH, or SUH). This binding, along with the co-activator Mastermind-like (MAML), results in a switch from a transcriptional repressor complex to an activation complex. Together, this binding complex ultimately activates expression of downstream target genes such as basic helix-loop-helix transcriptional repressors related to Hairy enhancer of split (Hes1, 5, 7), or to Hes-related with YRPW motif (Hey1, 2 and L), to affect many cellular processes including cell proliferation and differentiation.[3,4] While the NICD protein has the ability to function in an RBP-J-independent manner, non-canonical Notch signaling has yet to be implicated in skeletal development.

Notch signaling has emerged as an important regulator of skeletogenesis with multiple roles in somitogenesis, chondrogenesis, osteoblastogenesis and osteoclastogenesis. Several Notch signaling pathway components regulate somite segmentation, which underlies axial skeleton patterning. In addition, Notch pathway manipulation in the chondrogenic and osteogenic lineages in vivo demonstrates that Notch inhibits chondrogenic and osteogenic differentiation and growth in the axial and appendicular skeleton. In this chapter, we will discuss Notch pathway regulation of normal skeletal development and its role in congenital skeletal malformations and associated diseases.

Notch Pathway Regulation of Somitogenesis

The segmented pattern of the developing vertebral column is determined by the symmetric alignment of periodic condensations of paraxial mesoderm termed somites.[5] Somitogenesis, the organized formation of somites, is required for progenitor specification and amplification, as well as patterning of the developing axial skeleton. The formation of the first somite commences directly after gastrulation and new somites form every few hours until the species-appropriate number of somites is formed. Arising from the presomitic mesoderm (PSM), the epithelial derived somites are sequentially formed in condensed, bilateral pairs along the developing neural tube in an anterior to posterior direction. This spatially and temporally regulated formation occurs through cyclic oscillation of genes in the PSM, termed the somite segmentation clock, involving members of the Wnt, Fgf and Notch signaling pathways.[5-7] Somitogenesis results in a repetitious formation of evenly spaced and precisely sized somites along the midline that underlies proper vertebral development. After segmentation, somites are specified and differentiate into three distinct compartments, the dermatome, myotome and sclerotome. The epithelial dermatome generates the dorsal dermis (skin) while the myotome yields the musculature of the epaxial back muscles, hypaxial body wall muscle and skeletal limb muscle. The mesenchymal sclerotome, the precursor population of cells that will make up the axial

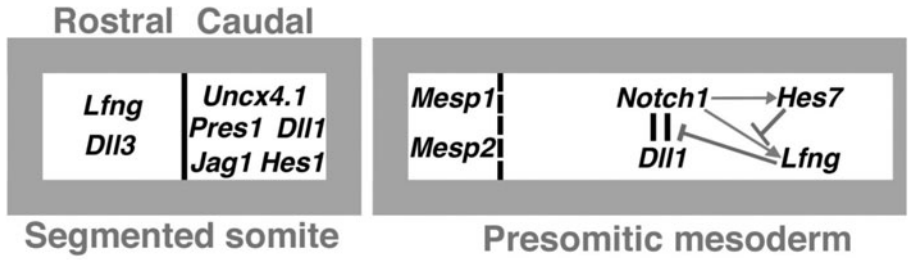


Figure 1. Notch pathway gene expression in somitogenesis. Notch pathway genes control somite formation, segmentation and polarity through variable and periodic expression. The somite segmentation clock consists of a negative feedback loop involving Notch signaling in the presomitic mesoderm (PSM) that regulates somite segmentation. Dll1 binds to Notch1 activating the transcription of *Hes7* and *Lfng*. Lfng then inhibits Dll1 and Notch1 binding while Hes7 represses transcription of *Lfng*. *Mesp1* are *Mesp2* are expressed in the rostral half of the PSM. The newly formed somites are polarized with differential expression of *Lfng* and *Dll3* in the rostral half while *Uncx4.1*, *Pres1*, *Dll1*, *Jag1* and *Hes1* are expressed in the caudal half of the developed somite. Please see text for references.

skeleton, gives rise to the chondrogenic and osteogenic vertebral column consisting of vertebral bodies, intervertebral discs, pedicles and ribs.[5] Thus, proper somitogenesis is required for appropriate formation of multiple skeletal elements of the developing vertebral column.

The initial condensation of somites is governed largely by Notch signaling. In the somite segmentation clock, Notch pathway components are expressed periodically in the PSM and undergo a remarkable spatial and temporal activation and inactivation during each segmental cycle to play a critical role in somite formation and polarity (Fig. 1). Notch pathway involvement in the segmentation clock has been largely elucidated through the investigation of animal models (Table 1). *Notch1* is expressed throughout the PSM and mice lacking *Notch1* display delayed and irregular somite segmentation with early embryonic lethality.[8,9] Mice lacking the Notch pathway effector, RBP-J, share a similar phenotype with *Notch1* nulls, apparent in irregular somite formation and segmentation.[10] Mouse mutants lacking the Notch pathway cleavage protein Presenilin2 exhibit no obvious defects in somitogenesis.[11] However, *Presenilin1* deficient mice demonstrate abnormal somite polarity while *Presenilin1/2* double mutant mice possess a more extreme somitogenesis phenotype with lack of segmentation and early embryonic lethality.[11,12] Through Notch activation, *Mesp1* and *Mesp2* genes are expressed in the PSM and mice lacking either gene exhibit somite segmentation defects.[13,14]

Notch signaling is not only necessary in somite formation, but also required for establishing rostrocaudal somite polarity. Mice deficient in Notch ligands Dll1 or Dll3, display somite segmentation and polarity defects with *Dll3* expressed in the rostral half and *Dll1* expressed in the caudal half of the developing somite.[15,16] The Notch pathway modulator, Lunatic Fringe (Lfng), exhibits oscillatory expression in the PSM via a negative feedback loop. Along with activator Mesp2, Notch1 activation leads to *Lfng* transcription, which then inhibits Delta ligand and Notch receptor binding.[5] This inhibition represses expression of *Lfng* during each segmental cycle to allow for period expression of Notch signaling and proper somite formation. Expressed in the PSM and the rostral half of the somite, *Lfng* mutant mice have defective somite segmentation and patterning.[17,18] Notch pathway downstream effector proteins Hes1, 5 and 7 also cycle in the PSM during somite

Table 1. Mouse mutants in Notch pathway genes associated with skeletal malformations

Mouse Mutant	Phenotype	References
Defective somitogenesis		
Notch1 null	Delayed & disorganized segmentation	8,9
RBP-J null	Delayed & irregular shaped somites	10
Presenilin1/2 null	Lack of somite segmentation	11
Mesp1 null	Irregular segmentation	14
Dll1 null	Segmentation & R/C[a] defects	15
Hes7 null	Segmentation, R/C, & sclerotome defects	19
Mesp2 null	Segmentation & sclerotome defects	13
Dll3 null	R/C & sclerotome defects	16
Lfng null	R/C & sclerotome defects	17,18
Presenilin1 null	R/C & sclerotome defects	12
Uncx4.1 null	Sclerotome defects	22
Col2a1Cre;Rosa^{Notch}	Sclerotome defects	23
Defective chondrogenesis		
Prx1Cre;Presenilin1$^{f/f}$/2$^{-/-}$	Shorter limb bones, increase of HCs	38
Prx1Cre;Notch1$^{-/f}$/2$^{f/f}$	Shorter limb bones, increase of HCs	38
Prx1Cre;Notch1$^{f/+}$/2$^{f/f}$	Shorter limb bones, increase of HCs	38
Prx1Cre;Notch1$^{f/f}$/2$^{f/+}$	Slight increase of HCs	38
Prx1Cre;RBP-J$^{f/f}$	Accelerated chondrogenesis; increase of HCs	33
Prx1Cre;Rosa^{Notch}	Decreased/delayed chondrogenesis; increased prolif.	33
Col2a1Cre;Rosa^{Notch}	Chondrodysplasia; decreased prolif. & diff.	23
Col2a1Cre;RBP-J$^{f/f}$	Increased prolif. and number of HCs	23
Col2a1Cre;Hes1$^{fl/fl}$/5$^{-/-}$	No skeletal phenotype	39
Defective osteoblastogenesis		
Col1a1(2.3-kb)-*N1ICD*	Osteosclerosis; increased osteoblast prolif.	47
Col1a1(3.6-kb)-*N1ICD*	Osteopenia; decreased number of osteoblasts	45
Col1a1Cre;Presenilin1$^{f/f}$/2$^{-/-}$	Osteoporosis	47
Col1a1Cre;Notch1$^{f/f}$/2$^{f/f}$	No skeletal phenotype	38
OsteocalcinCre;Notch1$^{f/f}$	No skeletal phenotype	45
Defective osteoclastogenesis		
LysmCre;Notch1$^{fl/fl}$/2$^{fl/fl}$/3$^{-/-}$	Increased osteoclast prolif. & activity	57
Col1a1Cre;Notch1$^{fl/fl}$	Increased active osteoclasts	57
Col1a1Cre;Presenilin1$^{f/f}$/2$^{-/-}$	Osteoporosis; increased number of osteoclasts	47
Col1a1(2.3-kb)-*N1ICD*	Osteosclerosis; decreased activated osteoclasts	47
Col1a1(3.6-kb)-*NICD*	Osteopenia; decreased osteoclasts per tissue area	45

[a] Abbreviations: R/C: rostrocaudal; HC: hypertrophic chondrocytes; prolif.: proliferation; diff.: differentiation

segmentation and repress their own expression. However, *Hes7* mutants are the only one of these that have disrupted somitogenesis including segmentation, rostrocaudal and sclerotome defects.[19] While *Hey1*, *2* and *3* (*L*) are all expressed in the PSM, mice lacking these factors individually fail to show any signs of defective somitogenesis.[20,21]

After somite formation and polarization, somites differentiate into the dermatome, myotome and sclerotome derivatives. Mesenchymal sclerotome cells condense and differentiate into chondrocytes to form a cartilaginous pre-skeleton in which some elements remain chondrogenic, while others are subsequently replaced by bone. Expressed in the caudal half of the somite, *Uncx4.1* was demonstrated to play a role in sclerotome formation and differentiation, with null mice exhibiting sclerotome-derived vertebral defects including the loss of pedicles, transverse processes and proximal ribs.[22] In addition, *Dll3* mutant mice exhibit disrupted sclerotome formation including defective vertebrae with fused and bifurcated ribs.[16] Furthermore, mutant mice that lack RBP-J in *Col2a1Cre*-expressing cells, including sclerotome precursors, exhibit malformed vertebrae. In contrast, *Col2a1Cre*-driven gain of N1ICD signaling results in loss of the vertebral column and accessory ribs, illustrating a critical role for precisely regulated levels of Notch1 signaling in axial skeleton development.[23] Collectively, these data show that components of the Notch signaling pathway regulate somite segmentation, patterning and differentiation into sclerotome to allow for proper axial skeleton development.

Notch Signaling Initiates Chondrogenesis while Repressing Differentiation

Chondrogenesis is a multi-step process that commences when pluripotent mesenchymal precursors commit to the chondrogenic cell lineage.[24,25] Mesenchymal derived chondrocytes originate from several sources. Craniofacial cartilage is derived from cranial neural crest cells, the sclerotome of the somites forms the axial skeleton and the limb skeleton is derived from lateral plate mesoderm. These mesenchymal precursor cells, under strict molecular regulation, proliferate and differentiate to form cartilage primordia that prefigure future skeletal elements.

There are two main types of cartilage in the developing body. Articular cartilage is permanent connective tissue that is located at the end of bones and in the cranium and functions to bear weight, allow for bone movement and is highly flexible.[25] Endochondral ossification, the process of appendicular skeleton bone formation, relies on chondrogenic precursor cells as an intermediate to bone development (Fig. 2).[24] Early condensed mesenchymal cells that are fated to become chondrocytes are arranged in a growth plate, also known as an epiphyseal plate and transition through a sequential series of chondrogenic zones. Chondrocytes in the resting/reserve zone are relatively quiescent and small and rounded in appearance with abundant ECM. This reserve population of chondrogenic cells transitions to the proliferative zone where they are flattened and align in parallel, longitudinal columns of dividing cells. These cells transition from proliferative chondrocytes to prehypertrophic chondrocytes, in which chondrocytes stop dividing and start to undergo hypertrophy. During hypertrophy, these cells increase their intracellular volume up to 10 fold prior to calcification.[26] Finally, the hypertrophic chondrocytes undergo apoptosis, providing the mineralized matrix as a scaffold for future bone development, osteogenesis. The cartilaginous scaffold is invaded by blood vessels, which is a necessary step in endochondral ossification, whereby osteoblasts are transported to the cartilage scaffold to form a calcified bone matrix.[27,28] Bone growth is an ongoing process that is

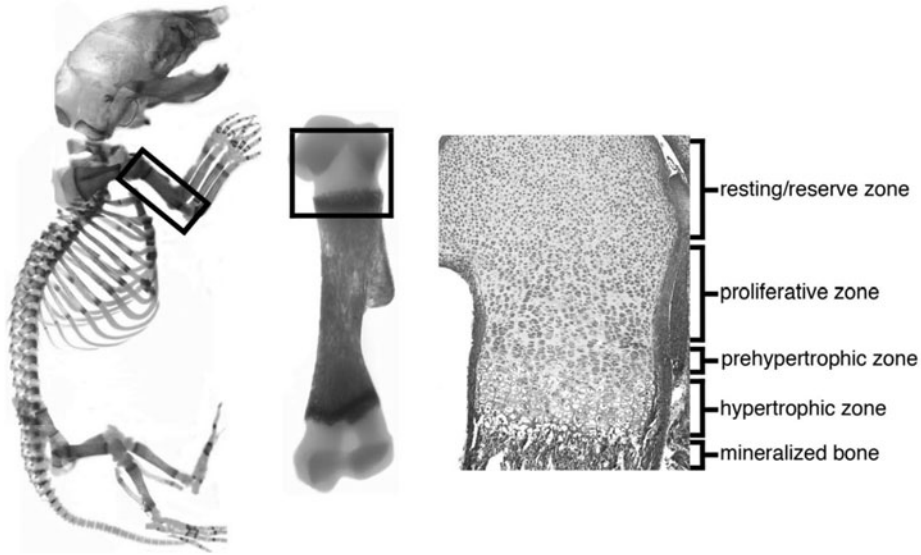


Figure 2. The progression of cartilage differentiation in endochondral bone formation. A whole-mount embryonic day (E)18.5 mouse skeleton stained with alcian blue (cartilage) and alizarin red (bone) demonstrates the distribution of cartilage and bone in the embryonic skeleton. The humerus bone (black box on the skeleton) was isolated and sectioned to illustrate the growth plate (black box around humerus) of the developing endochondral bone. Under strict molecular regulation, chondrocytes differentiate into different zones including the resting/reserve, proliferative, prehypertrophic and hypertrophic chondrocyte zones and finally undergo apoptosis to provide the scaffold for mineralized bone. Please see text for references. A color version of this figure is available at www.landesbioscience.com/curie.

driven primarily by the rate of hypertrophic chondrocyte production from the proliferating chondrocytes and ends with the cessation of puberty in humans.[25,29]

Notch signaling inhibits the process of chondrogenesis at multiple stages as demonstrated by multiple genetic approaches. In murine in vitro studies, Notch1 is expressed in early mesenchymal condensations where it promotes chondrogenic specification, but over-expression of N1ICD or Dll1 inhibits chondrogenic proliferation and differentiation.[30,31] Likewise, loss of Notch signaling, with the use of a gamma secretase inhibitor (DAPT) in a murine limb bud micromass culture system, results in enhanced differentiation of chondrogenic condensations and nodule formation.[32,33] In human cell culture studies, Jagged1 is required to initiate chondrogenesis, but Notch signaling must be terminated to allow for completion of chondrogenesis to occur.[34] Early mesenchymal condensation is regulated by the transcription factor sex determining region Y box 9 (Sox9), which is required for chondrocyte commitment and regulates chondrogenic proliferation and differentiation.[35] Studies in human mesenchymal stem cell cultures show that Notch-regulated transcription factors Hes1 and Hey1 bind to the Sox9 consensus binding site on the *Col2a1* promoter, thus inhibiting Sox9 binding and repressing chondrogenic differentiation (Fig. 3).[36] Together, these studies illustrate that Notch signaling is required for initial formation of chondrogenic precursors, but then represses chondrogenic differentiation through the inhibition of Sox9.

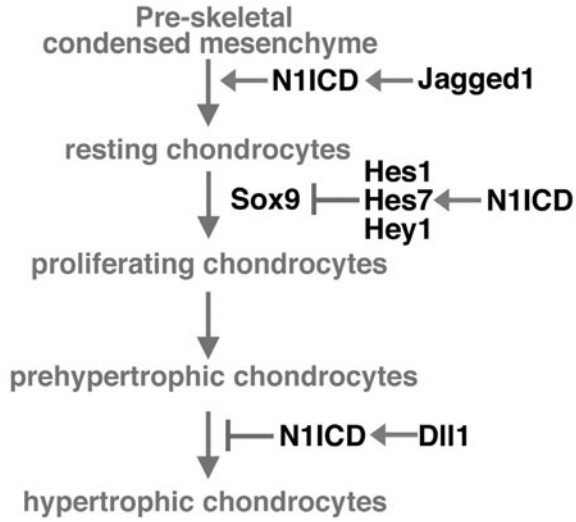


Figure 3. Notch pathway regulation of chondrocyte differentiation. Notch signaling has multiple roles at different stages of chondrogenic differentiation. Jagged1 and N1ICD promote the commitment of preskeletal mesenchyme to the chondrocyte lineage. However, N1ICD inhibits the transition from resting chondrocytes to proliferating chondrocytes through activation of Hes1 and Hey1 to inhibit the transcriptional activity of Sox9 or via activation of Hes7 to inhibit *Sox9* gene expression. In addition, Dll1 mediates inhibition of prehypertrophic to hypertrophic chondrocyte differentiation. Please see text for references.

In addition to in vitro studies, the suppressive function of Notch signaling in chondrogenic differentiation has been elucidated through in vivo models (Table 1). In the chick embryo, increased expression of *Dll1* was shown to negatively regulate the transition from prehypertrophic to hypertrophic chondrocyte, causing skeletal abnormalities including truncated skeletal elements that lack calcification.[37] Loss of Presinilin1 and Presinilin2 in murine calvaria and throughout the limb mesenchyme driven by *Prx1Cre* results in accumulation of hypertrophic chondrocytes in the growth plate and an increase of trabecular bone, leading to skeletal malformations.[38] In addition, loss of both Notch1 and Notch2 with *Prx1Cre* results in a similar phenotype with shortened endochondral bones and an increase of trabecular bone density. Loss of Notch2 alone results in a similar phenotype, suggesting that Notch2 is the predominant receptor in endochondral bone formation.[38] Furthermore, conditional over-expression of N1ICD with the use of *Prx1Cre* suppresses chondrogenic differentiation as indicated by reduced expression of *Sox9*, *Col2a1* and *Aggrecan*, which are markers of resting/reserve chondrocytes.[33] The reverse is true for conditional loss of RBP-J with *Prx1Cre*, resulting in accelerated chondrogenesis with increased expression of *Col2a1* and *Aggrecan*.[33] Interestingly, loss of RBP-J rescued the *N1ICD* gain-of-function phenotype in a compound mutant mouse. This illustrates that Notch signaling in skeletal development is RBP-J-dependent, indicating canonical Notch signaling.[33] Together these results demonstrate that Notch signaling in early limb mesenchyme negatively regulates chondrogenic differentiation and that dysregulation of Notch signaling results in chondrogenic malformations.

Notch signaling was also investigated specifically in the differentiated chondrocyte lineage utilizing *Col2a1Cre* mice. Over-expression of N1ICD in cartilage progenitors using *Col2a1Cre* results in decreased chondrogenic proliferation and differentiation. These mice have severe skeletal malformations, including a reduction of the appendicular skeleton and complete lack of some axial skeletal elements, resulting in perinatal lethality.[23] In this study, increased N1ICD resulted in decreased chondrocyte-specific gene expression at multiple stages of chondrogenic differentiation. Expression of early markers, *Sox9* and *Aggrecan*, were significantly decreased as was the prehypertrophic chondrocyte marker Indian hedgehog (*Ihh*). Hypertrophic chondrocyte markers *Col10a1* and *runt-related transcription factor 2* (*Runx2*) also were decreased, potentially through Hes7 inhibition of *Sox9* gene expression and transcriptional activity.[23] Intriguingly, the N1ICD gain-of-function mice with decreased *Sox9* expression phenocopy cartilage-specific Sox9 loss of function mice, which resemble the human skeletal dysmophic syndrome, campomelic dysplasia.[35] In further investigation, conditional loss of RBP-J utilizing *Col2a1Cre* results in increased chondrogenic proliferation and hypertrophic chondrocytes with perinatal lethality.[23] Interestingly, conditional loss of Notch1 in the chondrogenic lineage did not have as striking a skeletal phenotype as the RBP-J loss-of-function mice at birth, further suggesting that Notch2 may be the predominant Notch receptor in endochondral bone formation (Mead and Yutzey, unpublished data). Through the investigation of Notch downstream target genes, *Hes1/5* double mutant mice in the *Col2a1Cre* lineage show no overt skeletal defects, pointing to either redundancy or another downstream Notch mediator regulating the timing of chondrogenic differentiation.[39] Overall, Notch signaling has been shown in vitro and in vivo to suppress chondrogenic differentiation (Fig. 3).

Notch Inhibition of Osteoblast Differentiation

Bone is mineralized connective tissue that is the main component of the skeletal system and provides strength and support for the body. There are two types of bone formation, endochondral ossification (described above) and intramembranous ossification, which forms the flat bones of the body and involves the differentiation of cells within the mesenchymal condensations directly into bone.[40] Osteoblasts, or bone forming cells, share a common progenitor with chondrocytes and are also derived from multipotent mesenchymal cells.[41] After osteoblast lineage induction, the precursor population proliferates to expand and subsequently undergoes maturation and finally mineralization. Residing near the bone surface, osteoblast precursor cells proliferate and commit to the osteoblast lineage. These pre-osteoblasts undergo matrix maturation through the expression of early markers of osteogenic differentiation including *Col1a1*, *alkaline phosphatase* (*Alp*) and *Runx2* and subsequently mineralize and express late makers of osteoblast differentiation including *Osteocalcin* (*Ocn*).[40] Mature and functional osteoblasts, or osteocytes, provide mechanical support and regulate mineral deposition.

Notch regulation of osteoblast commitment and differentiation has been well documented. In vitro studies show that Notch signaling suppresses osteoblastic differentiation through the inhibition of osteogenic markers *Alp*, *Ocn*, *Col1a1* and *Runx2*, resulting in suppression of calcification.[42-45] In stromal cells, N1ICD signaling inhibits osteoblastogenesis by Hes1-mediated suppression of Wnt/β-Catenin signaling, indicating an antagonistic relationship between the pathways in osteogenesis (Fig. 4).[46] Furthermore, repression of osteoblast differentiation is also mediated through

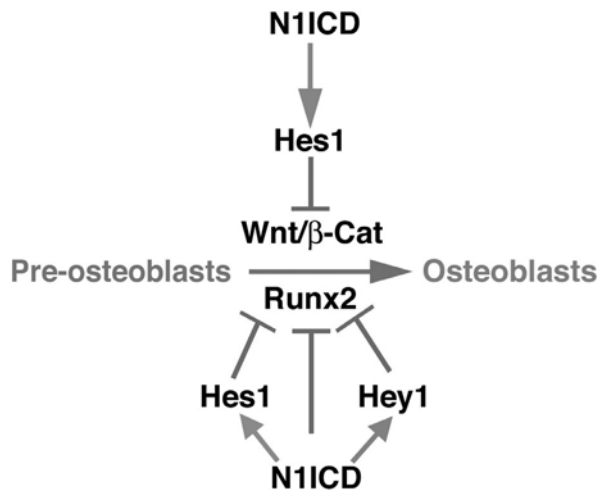


Figure 4. Notch pathway inhibition of osteoblastogenesis. N1ICD negatively regulates osteoblast formation through Hes1-mediated repression of Wnt/β-Catenin or via Hes1- and Hey1-mediated or N1ICD direct repression of Runx2. Please see text for references.

Notch1 itself or by Notch downstream effectors Hes1 and Hey1 binding directly to and inhibiting Runx2 transcriptional activity.[38,44,47] Runx2 is considered the master regulator of osteoblast differentiation. Forced expression of Runx2 in non-osteoblast cells is sufficient to induce the expression of many osteoblast genes while Runx2 null mice lack osteoblasts, resulting in defective osteoblast differentiation and no endochondral or intramembranous bone formation.[48-50] Thus, Notch-mediated repression of Runx2 transcriptional activity provides evidence for a direct mechanism of osteoblastogenesis inhibition (Fig. 4). In disagreement, two studies show that increased Notch signaling in MC3T3 cells stimulates osteoblast differentiation through the induction of calcific nodules.[51,52] It is likely that the differing results could be due to cell culture conditions or the cell lines utilized and that the timing and levels of Notch signaling determine its effects on osteogenic gene induction.

While there have been controversial in vitro results, recent in vivo data have helped clarify the role of Notch signaling in osteoblastogenesis (Table 1). Loss of Presenilin1 and Presenilin2 in the osteoblast lineage results in overall loss of bone mass and age-related osteoporosis.[47] Over-expression of N1ICD under the control of the 3.6kb *Col1a1* promoter, expressed in early osteoblast precursors, results in runting from an overall decrease of bone volume leading to osteopenia.[45] N1ICD over-expression with the 2.3kb *Col1a1* promoter, expressed in mature osteoblasts, also results in skeletal dysfunction with progressive growth retardation, but due to increased proliferation of immature osteoblasts leading to increased bone mass and osteosclerosis.[47] In this setting, Notch inhibits terminal osteoblast differentiation of committed progenitors, which allows for proliferation of immature osteoblasts. Together, activation of N1ICD inhibits osteoblast differentiation in both cases, but the resulting phenotype is dependent on the time of inhibition. Loss of Notch1 alone or of Notch1 and Notch2 together in differentiated osteoblasts did not result in skeletal abnormalities, indicating

that Notch does not regulate mature osteoblast homeostasis, but inhibits osteoblast precursor differentiation during development.[38,45] Collectively, these in vitro and in vivo studies illustrate Notch inhibition of osteoblast differentiation at multiple stages of osteoblastogenesis.

Notch Pathway Suppression of Osteoclastogenesis

Osteoclasts are derived from hematopoietic cells of the monocyte/macrophage lineage and provide the unique function of bone resorption.[53] In conjunction with bone producing osteoblasts, osteoclasts maintain the skeletal system in homeostasis. Osteoclastogenesis commences when a subset of macrophages commit to the osteoclast lineage, proliferate, differentiate and reabsorb bone. After osteoclast lineage determination, macrophage colony-stimulating factor (M-CSF) is required for pre-osteoclast proliferation and survival.[54] However, the differentiation of osteoclasts is essentially regulated by signaling interaction with osteoblasts. Osteoblasts express M-CSF and receptor activator of nuclear factor kappa B ligand (RANKL) to promote macrophage commitment to the osteoclast lineage. RANKL promotes osteoclastogenesis through the stimulation of a transcription factor complex including nuclear factor of activated T-cells 1 (NFATc1), while also later promoting bone resorption through the induction of a bone reabsorbing complex including the RANK receptor.[53] Similar to RANKL, osteoprotegerin (OPG) is produced by osteoblasts and competes with the RANK receptor for RANKL, acting as a decoy receptor effectively modulating osteoclast production.[55] Therefore, the balance between the osteoclast stimulator, RANKL and the osteoclast inhibitor, OPG, determines the amount and rate of osteoclast production.

Notch signaling negatively regulates osteoclastogenesis as demonstrated by in vitro and in vivo manipulation of Notch signaling directly in osteoclasts and indirectly in osteoblasts (Table 1).[42,47,56,57] Activated Dll1 inhibits osteoclast formation in hematopoietic cells, while constitutively active Notch1 reduces *M-CSF* and enhances *RANKL* and *OPG* gene expression, resulting in an overall reduction of osteoclast formation in stromal cells.[56] In a further study, constitutively active N1ICD expression in mesenchymal cell lines inhibits osteoclastogenesis via inhibition of *RANKL* expression.[42] In agreement, Jagged1 inhibits osteoclastogenesis in bone marrow macrophages while Notch1 and Notch3 loss of function in vivo in the osteoclast lineage directly promotes osteoclast formation with increased cell proliferation.[57] Furthermore, loss of *Notch1* in the osteoblast lineage increases *RANKL* expression and decreases expression of *OPG*, therefore, indirectly promoting osteoclast formation.[57] In addition, conditional loss of Presenilin1 and Presenilin2 in the osteoblast lineage results in increased osteoclasts due to a reduction of *OPG* expression, leading to osteoporosis.[47] Overall, these studies show that Notch signaling negatively regulates osteoclast formation and proliferation (Fig. 5). Recently, some controversy on the role of Notch signaling in osteoclastogenesis has arisen with a study showing that RANKL has the potential to induce Jagged1 and Notch2 in bone marrow macrophages, while loss of Notch signaling or introduction of Notch2 shRNA suppresses RANKL-induced osteoclastogenesis. Likewise, Notch2 was shown to bind to the *NFATc1* promoter and drive its expression, also resulting in increased osteoclast formation.[58] It is plausible that Notch signaling regulates multiple stages of osteoclastogenesis to either activate or repress osteoclast formation and activity. However, additional studies are required to reconcile these differing roles of Notch signaling in osteoclastogenesis.

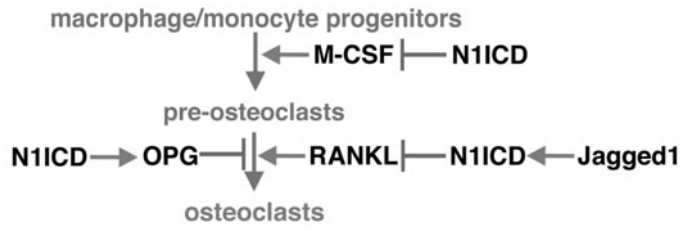


Figure 5. Notch pathway repression of osteoclastogenesis. N1ICD negatively regulates osteoclast formation at multiple steps of osteoclast maturation. N1ICD inhibits the differentiation of monocyte/macrophage lineage cells to commit to the osteoclast lineage via repression of M-CSF. Furthermore, N1ICD inhibits *RANKL* while activating *OPG* gene expression, both actions leading to the inhibition of osteoclast formation. Please see text for references.

NOTCH PATHWAY ASSOCIATED SKELETAL DISORDERS

The Notch signaling pathway is essential for proper skeletal development and dysregulation can lead to malformations and disease. Consistent with critical roles of Notch signaling in somitogenesis, chondrogenesis and osteogenesis, there are a large number of human skeletal diseases that are associated with dysregulation of Notch signaling. Mouse models have been useful in defining the cellular and molecular basis of these human skeletal congenital defects related to altered Notch signaling (Table 2).

Defective somitogenesis can result in many developmental disorders of the axial skeleton including spondylocostal dysostosis. Spondylocostal dysostosis (SD) is characterized by trunk dwarfism as a result of disrupted vertebral and rib anomalies, including multiple hemivertebrae, butterfly vertebrae, rib fusions, deletions and malalignment.[59] Individuals affected with this disorder have a protrusion of their abdomen and short stature throughout adulthood and often exhibit scoliosis or kyphoscoliosis.[60] Currently, there are four subtypes of familial SD linked to autosomal recessive alleles of Notch pathway genes involved in the somite segmentation clock, rostrocaudal patterning and sclerotome formation. SD1 is caused by protein truncations and missense mutations in the human Notch ligand *DLL3*. The phenotype of these patients, including a short trunk and abdominal protrusion, resembles the mouse *pudgy* mutation, a spontaneous *Dll3* gene mutation.[16,60,61] Mutations associated with SD2 are attributed to the Notch pathway gene *MESP2*. In agreement with *Mesp2* null mice, individuals affected with SD2 possess a milder phenotype than SD1, but still have significant vertebral malformations.[13,62] SD3 is caused by mutations in Notch regulated human gene *LFNG* that leads to a phenotype that not only encompasses the vertebral malformations of Dll3 and Mesp2 deficient mice, but also includes digit abnormalities.[63] Recently, mutations in *HES7* have been identified as SD4.[64,65] In agreement with *Hes7* mutant mice, these patients exhibit vertebral segmentation defects affecting the axial skeleton.[19] Based on studies in mice, it is likely that additional Notch pathway genes will be identified with a SD spectrum disorder. Recent studies demonstrated that N1ICD expression in the sclerotome results in a murine SD phenotype with a severe reduction of the vertebral column as well as abnormal rib morphology.[23] Murine Notch1 has been shown to interact with Dll3 resulting in vertebral and craniofacial malformations and interacts with Mesp2, Lfng and Hes7 in somitogenesis.[13,19,66,67] However, to date no human *NOTCH1* mutations have been linked to SD malformations.

Table 2. Human skeletal diseases caused by Notch pathway mutations

Syndrome	Gene	Skeletal Manifestation	Reference
Spondylocostal dysostosis			
	Type 1 = *DLL3*	Vertebral defects, hemivertebrae, & rib fusions	60
	Type 2 = *MESP2*	Thoracic vertebral defects only	62
	Type 3 = *LFNG*	Vertebral defects, rib defects, & digit abnormalities	63
	Type 4 = *HES7*	Vertebral, rib, & cranial defects	64,65
Alagille syndrome			
	JAGGED1	Butterfly vertebrae, craniofacial, & digit defects	68,69
	NOTCH2	Characteristic facial features	71

Alagille syndrome (AGS), caused by mutations in the Notch ligand *JAGGED1* and to a lesser extent in the *NOTCH2* receptor, is an embryonic developmental disorder involving many organs including the liver, heart, eye and skeleton.[68-71] The skeletal defects associated with AGS consist of butterfly vertebrae, craniofacial defects and digit abnormalities, all of which point to a primary defect in chondrogenesis. Children with AGS have a short stature with decreased bone area and mass relative to their peers, which puts them at a greater risk for fractures and osteoporosis in childhood and adulthood.[72] While *Jagged1* null mouse models recapitulate some organ abnormalities, mice that are heterozygous for *Jagged1* and a *Notch2* hypomorphic allele ($J1N2^{+/-}$) exhibit many clinically relevant features of the syndrome.[73,74] However, $J1N2^{+/-}$ mice exhibited no skeletal abnormalities associated with AGS, potentially evoking another Notch pathway genetic modifier in the AGS-associated skeletal malformations.

Many additional skeletal malformations have been associated with dysregulated Notch signaling in mice. Thus, in vitro and in vivo manipulations of the Notch pathway that result in skeletal dysfunction in mice provide insight into potential candidate genes for inherited human skeletal malformations. There are numerous Notch pathway mouse mutants that have defective axial skeleton development involving the vertebral column and cranium. Scoliosis, a congenital malformation of the vertebral column, occurs in $Dll3^{+/-}Notch1^{+/-}$ double heterozygous mutant mice.[66] Recently, there have been a number of studies that point to a primary contribution of altered Notch signaling in the generation of craniofacial defects. $Dll3^{+/-}Notch1^{+/-}$ mutant mice, along with mice over-expressing *N1ICD* in limb bud mesenchyme and in chondrocytes, exhibit craniofacial malformations.[23,33,66] In addition, gain or loss of Notch signaling in the neural crest lineage results in craniofacial defects including micrognathia and exencephaly (Mead and Yutzey, unpublished).

Deregulated chondrogenesis often results in chronic illnesses and bone growth failure. Chondrodysplasia and diseases involving decreased bone mineralization, including osteopenia and the more severe osteoporosis, have been linked to defective Notch signaling.[23,38,45,47] Excessive bone resorption by osteoclast activity results in pathologic bone destruction including osteoporosis, while inefficient or arrested bone resorption results in a disease of increased bone mass known as osteopetrosis. While Notch loss of function in the osteoclast lineage results in osteoporosis, the complimentary experiment of over-expression of *N1ICD* in the osteoclast lineage, which would be predicted to

lead to osteopetrosis, has yet to be performed.[57] Notch signaling has also been linked to the degenerative joint disease, osteoarthritis. Several reports have shown that Notch signaling is dysregulated in osteoarthritis, but the role of Notch signaling in osteoarthritis proliferation and progression are not clear and further investigation is warranted.[75-77]

Recently, Notch signaling has been implicated as an activator in the most common bone tumor, osteosarcoma. In human samples, *Jag1*, *Dll1*, *Notch1*, *Notch2*, *Hes1* and *Hey1* were all reported to have increased expression in osteosarcoma tissue, as compared to normal bone.[78,79] In vitro and in vivo loss of Notch signaling decreased osteosarcoma proliferation, resulting in decreased tumor growth.[78,80] Furthermore, the Notch target gene *Hes1* has been shown to activate osteosarcoma invasion and metastasis, while the non-canonical Notch target gene *Deltex1* exhibits reciprocal inhibition with *Hes1* to regulate Notch signaling in osteosarcoma tumor growth, invasion and metastasis.[79,81,82] Cancer has been shown to recapitulate development in terms of increased proliferation and differentiation involving similar molecular mechanisms of development. Notch signaling is known to regulate cell proliferation and differentiation in multiple systems, including skeleton development. Therefore, increased knowledge on the role of Notch signaling in skeletogenesis could be applied in the targeting of cancer and development of new therapeutic avenues.

CONCLUSION

Notch signaling regulates skeletogenesis through the control of somitogenesis, chondrogenesis, osteoblastogenesis and osteoclastogenesis. In many organ systems, including the developing skeletal system, Notch signaling has been shown to maintain cells in a primitive, undifferentiated state through the regulation of proliferation and inhibition of cell differentiation. The Notch signaling pathway regulates somite formation, patterning and differentiation and suppresses chondrogenic and osteoblastic differentiation, while negatively regulating osteoclast formation and proliferation. Too much or too little Notch signaling in skeletal precursor populations can result in skeletal malformations, indicating that precise levels of Notch signaling are required for proper skeletogenesis.

While the inhibitory role of Notch signaling in skeletogenesis has become apparent through multiple in vitro and in vivo studies, many questions remain unanswered. The canonical Notch pathway has been shown to regulate skeletogenesis, however, little is known of RBP-J-independent noncanonical Notch signaling in relation to the skeleton. Furthermore, it is critical to understand the role of Notch signaling at specific time points in embryonic and adult development and disease. Conditional activation and inhibition of Notch signaling in skeletogenesis with the use of murine tamoxifen-inducible Cre expression lines such as *Col1a1CreER*, *Col2a1CreER* and *Prx1CreER* will allow for temporal and spatial restricted expression or deletion of Notch pathway genes.[83-85] These conditional mouse lines will allow the study of the role of Notch signaling in postnatal and adult chondrogenesis and osteogenesis to clearly investigate roles in cartilage and bone homeostasis and density. Furthermore, intramembranous bone development, including craniofacial bone development, has been largely overlooked in recent research.

Development of therapeutics related to Notch-associated skeletal disease will require more specific determination of ligand and receptor interactions. While there are many studies investigating the role of Notch signaling in skeleton development, there has not been extensive information on conservation of Notch ligand to receptor to

downstream target linkage, which is critical to reveal therapeutic potential. In addition, the ability to demonstrate Notch pathway synergy and antagonism of other major pathways required in skeletogenesis is critical to develop translational drug targeting mechanisms for diseases involving defective cartilage and bone. The Notch pathway has been implicated in many skeletal diseases and research going forward will help identify potential therapeutic interventions.

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CHAPTER 10

NOTCH SIGNALING AND THE DEVELOPING SKIN EPIDERMIS

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Abstract: The innermost (basal) layer of the skin epidermis consists of proliferative progenitors which give rise to multiple differentiating layers providing a barrier that keeps the inside of the body moist and protects the body from outside assaults by physical, environmental and biological factors. The epidermis is maintained throughout life through the proliferation of stem cells and differentiation of their progeny. Notch signaling pathway is a highly conserved molecular network that plays an essential role in cell fate determination during embryogenesis and also in postnatal life. Data from ongoing studies indicate that Notch signaling orchestrates the process of epidermal differentiation and proliferation through the sequential activity of different Notch ligands, receptors and downstream pathways.

INTRODUCTION

The most important function of the skin is to generate a shield preventing desiccation, infection and damage by toxic agents. The epidermis is a stratified epithelium that constitutes the outer layer of the skin and is maintained throughout adult life by the ability to self-renew under both homeostatic and injury conditions through the activity of stem cells that lie in specific locations.[1] The major barrier resides within the external layers of the epidermis which are continually shed from the surface of the skin and are renovated through the proliferation of cells in the basal layer and terminal differentiation of keratinocytes with formation of stratum corneum.[2] The process of terminal differentiation begins when basal cells concomitantly withdraw from the cell cycle and lose their ability to adhere to the basement membrane. In the intermediate spinous layers the cells enforce

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a durable cytoskeletal framework of keratin filaments to ensure the mechanical strength necessary to resist physical trauma. In the granular layers, keratins are cumulated into microfibrils by their association with filagrin, lipids are produced inside lamellar bodies and a cornified envelope is assembled by incorporation of precursor proteins under the plasma membrane. As the cell membrane disintegrates, the calcium influx activates the keratinocyte specific enzyme transglutaminase I to irreversibly cross-link the cornified envelope proteins and create a tough insoluble sac that surrounds the keratin fibers. Finally, lipids are extruded into the intercellular space onto the cornified envelope. This barrier, once built, is analogous to bricks and mortar, with the keratin macrofibrils and the cornified envelopes forming the bricks and the extruded lipids the mortar.[3]

By definition, adult stem cells have the ability to both self-renew and produce differentiated progeny.[4] In normal skin homeostasis, there are currently two proposed mechanisms to explain how stem cells provide the continuous supply of cells. The classic theory that after division of a stem cell one of the daughter cells develops into a transit-amplifying progenitor cell,[5] and an alternative mechanism where after division of a stem cell one of the daughter cells remains a stem cell and the other without further rounds of cell division compels to differentiate.[6]

In the quest to understand the biology of epidermal stem cells, research has focused on the main questions of interest: where are they located, what markers do they express and which signaling pathways regulate cell fate decisions. Evidence suggests that there are multiple populations of epidermal stem cells[7,8] that are responsible for replacing the differentiated cells of the interfollicular epidermis, hair follicles and sebaceous glands. In recent years there has been considerable progress in identifying the signaling pathways that regulate skin homeostasis and thus knowledge in this area has greatly increased. Several evolutionary conserved pathways have been shown to be important for embryonic skin development, epidermal stem cell maintenance, differentiation and lineage commitment including the Notch, Wnt/β-catenin, c-myc and p63 pathways[9-11] and there is emerging evidence of intersection between them.

NOTCH SIGNALING

The Notch signaling pathway is a highly conserved molecular network that, depending on the cellular and tissue context, as well as in relation to the contemporary crosstalk with other signaling systems, acts through the regulation of cell proliferation, migration, differentiation and apoptosis.[12-14] Notch genes encode single–pass, heterodimeric transmembrane receptors involved in cell-to-cell developmental interactions. These interactions are mediated by signals exchanged between contiguous cells expressing Notch ligands that bind and activate adjacent Notch receptors. There are different, although homologous Notch receptors present in different organisms. For example, while in *Drosophila melanogaster* there is only one receptor (Notch) and two ligands (Delta and Serrate), in mammals there are four receptors (Notch1 to Notch4)[15-17] and five ligands divided into two families (Jagged-1 and -2, Delta-like 1,3,4) which differ because of the presence in Jagged ligands of a cysteine-rich domain.[18-20] Although these four Notch receptors differ in their extracellular and cytoplasmic domains, they exhibit remarkable similarity. The extracellular domain of Notch includes a variable number of epidermal growth factor (EGF)-like repeats, with the function of ligand binding and three cysteine-rich Lin/Notch repeats that prevent signaling in the absence of the ligand. The cytoplasmic

region of Notch which conveys the signal to the nucleus contains a regulation of amino-acid metabolism (RAM) domain, six ankyrin repeats, two nuclear localization signals (NLS), a transactivation domain (TAD) and a proline-glutamate-serine-threonine rich (PEST) sequence. Notch signaling is initiated by receptor-ligand interaction between neighboring cells, which renders the Notch receptor susceptible to a first proteolytic cleavage by TACE (TNF-α-converting enzyme) and subsequently, to a second intramembranous cleavage by γ-secretase. This enzymatic processing results in the release of the Notch intracellular domain (NICD) from the plasma membrane, which may translocate to the nucleus. Once there, it associates with the DNA-binding protein RBP-Jk (also known as CSL, [acronym for CBF1, Su(H) and LAG-1][21] to generate a transactivation complex. The co-activator Mastermind is recruited to the complex and initiates transcription of downstream target genes including Hes and Hey of the basic helix-loop-helix family genes that are normally suppressed in the absence of Notch activity.[22] The Hes family repressor antagonizes the expression of a wide variety of activator-type basic helix-loop-helix transcription factors, including Asc1, Atoh1 and Neurog3.[23] Activated Notch in its free form is unstable and quickly degraded, an event that facilitates the regulation of inappropriate activation. Although the cascade of Notch signaling is relative simple, its role and the activation of downstream target genes in a different tissue is often unpredictable, at least in part because of its ability to contribute to diverse biological processes.[24]

Robust evidence has been accumulated showing that the evolutionarily conserved Notch pathway, which functions broadly in specifying cell fates during embryogenesis and adult life, has a key role in linking the control of epidermal differentiation and proliferation.

LOCALIZATION OF NOTCH RECEPTORS AND LIGANDS IN EPIDERMIS

In normal human skin, the Notch receptors and ligands are abundantly expressed in keratinocytes within the epidermis. Notch1 is primarily confined to keratinocytes in the lower and mid level epidermal layers. Notch2 distribution is limited to keratinocytes in the basal layer and Notch3 primarily is limited to keratinocytes in mid and upper layers of epidermis near the granular cell layer. Notch4 is primarily localized in the suprabasal layers. Jagged1 is expressed suprabasally and involves multiple cell layers, whereas Jagged2 is expressed basally.[25-27] By in situ hybridization Delta-like 1 has been detected in the basal layer of fetal and adult human interfolicular epidermis (IFE) and antibody staining suggests that Dll1 expression is highest in stem cell clusters.[28,29] The results of studies that have examined Notch pathway activation show that NICD is detected in few basal cells of the IFE and throughout the suprabasal layers and Hes1, the predominant target of Notch signaling in keratinocytes, is expressed suprabasally.[30-32]

GROWTH ARREST AND ADHESION

Epidermal homeostasis is based on the proper control of proliferation and differentiation programs within the different cells of epidermis.[33] Keratinocytes that undergo terminal differentiation withdraw from the cell cycle. Induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ occurs as one of the early steps in keratinocyte differentiation and contributes to growth arrest of these cells.[34] Rangarajan et al found that activated Notch1 and not Notch2 expression, triggers direct cell cycle withdrawal of mouse

primary keratinocytes by induction of $p21^{WAF1/CIP1}$ expression through RBP-Jk dependent transcription.[35] Increased Notch1 can also induce $p21^{WAF1/CIP1}$ expression in indirect RBP-Jk dependent mechanisms, as p21 activation has also been shown to be dependent on positive regulation of NFAT activity by activated Notch1, given that interaction between Notch1 and calcineurin –NFAT signaling is shown.[36]

An important function of Notch signaling in the epidermis is the regulation of cell adhesion, as the location of a stem cell in particular sites within the epidermis determines the signals received from the local microenviroment which direct the cell to self-renew, or differentiation along distinct lineages.[5,37,38] Studies in vivo show that loss of basal genes expression, such as integrins allowing basal cells to detach, occurs concomitant with NICD1 induction,[30,35] but there is also evidence that NICD can activate integrin-ligand binding activity without affecting integrin levels.[39] Estrach et al showed that Jagged1 and Dll1 have different effects on integrin expression.[40]

Reduced extracellular matrix adhesion is an important stimulus for terminal differentiation of cultured keratinocytes,[41] and this poses the possibility that Notch ligand-specific effects on adhesion could explain why Jagged1 differs from Dll1 in its effects on differentiation.[40] High Dll1 expression promotes keratinocyte cohesiveness and the cohesive effects of Dll1 in keratinocytes depend on the interaction of the PDZ-binding domain with syntenin. In addition, syntenin regulates endocytosis and reduces Dll1 internalization so mutation of the Dll1 PDZ domain or knockdown of syntenin induces rapid internalization of Dll1, which contributes to stimulation of Notch signaling.[42] Syntenin potentially could link Dll1 and integrins through its ability to bind syndecans, which in turn modulate integrin-mediated adhesion.[43,44]

Notch can also modulate keratinocyte adhesion by indirect mechanisms through the small GTPases of the Rho/Rac and Cdc42 families and their respective effectors ROCK2 and MRCKα.[45-47] Microarray analysis and RT-PCR confirm that expression of Rock2 and MRCKα is suppressed by activated Notch1 expression in human keratinocytes. Combined knockdown of Rock2 and MRCKα reduces integrin expression and cell motility, while upregulation suppresses differentiation and expands the stem cell compartment.[48]

NOTCH AND TERMINAL DIFFERENTIATION

The key point of the epidermal stratification is the spinous cell and the different missions that they have to carry out simultaneously such as: suppression of genes, particularly expressed in basal cells; upregulation of specific genes needed for suprabasal cell differentiation; maintainance of their immature and proliferative status; prevention of premature terminal differentiation; and initiation of the terminal differentiation program to differentiate into granular cells. Commitment to terminal differentiation consists in withdrawing from the cell cycle and suppressed expression of basal integrins, p63 and keratins K5/K14.[49] When an epidermal cell exits the basal layer and enters the first suprabasal layer, it induces expression of the 'early markers' which are keratin 1/10 and involucrin. As cells transit to the granular layers they express the 'late markers', the cornified envelope protein loricrin and the keratin-bundling protein filagrin.[50,51]

Ongoing studies have proven that Notch signaling plays multiple and apparently contradictory roles in the coordination of all these tasks for proper stratification during epidermal development in a Hes1-dependent or a Hes1-independent manner.[26,30,35,52] Blanpain et al showed that basal gene repression is dependent on canonical Notch/RBP-Jk signaling

and does not require Hes1.[30] In vitro and in vivo analyses agree that Notch signaling has a crucial role in the determination of the spinous cell fate as activation of Notch signaling in the spinous cells promotes fate determination by initiating genetic programmes that are necessary for suprabasal differentiation in which Notch activation induces spinous specific genes such as keratin 1/10, but there is discordance if its happens via a Hes1-dependent or independent mechanism.[30,52] It is intriguing that Notch1 signaling has seemingly contradictory roles in the spinous layers to both promote and prevent the granular differentiation, a fact that poses the question of how a single pathway can have such controversial actions in the same location. It seems that Notch signaling in a Hes1-dependent manner represses *Ascl2*, an achate/scute-related basic helix –loop-helix (bHLH) transcriptional activator to maintain spinous cells at early stages of epidermal development by preventing premature granular differentiation and simultaneously Notch1 signaling activates *Ascl2* expression to stimulate spinous differentiation and to promote granular cell fate, independent of the Notch downstream transcriptional factor Hes1.[52]

$p21^{WAF1/CIP1}$ was originally identified as a downstream mediator of p53-induced growth arrest and exerts anti- or proapoptotic functions that are cell type and context dependent. In addition, it has the potential of physically associating with transcription factors and coactivators, modulating their function. As mentioned, Notch1 signaling causes direct positive regulation of p21 expression through RBP-Jk dependent transcription, which promotes exit from the stem cell compartment. $p21^{WAF1/CIP1}$ also acts as a key mediator of Notch signaling in downsteam pathways (Wnt) and has an inhibitory role in the late stages of differentiation that does not result from its effects on the cell cycle.[53]

Okuyama et al with in vitro studies have proposed that Notch1 signaling by activating caspase 3 leads to decreased proliferation and increased differentiation of embryonic keratinocytes through activation of PKC-δ, but Blanpain et al failed to find support for canonical Notch signaling in controlling caspase 3 activity in vivo.[54,30]

DELETION OR ACTIVATION OF NOTCH PATHWAY COMPONENTS

Given the number of Notch receptors and ligands expressed in epidermis and the implication of Notch signaling in epidermal differentiation, the complete inactivation of signaling which is obtained only by targeting common Notch components such as γ-secretase, RBP-Jk or Mastermind like has interesting effects. It has been seen that, lacking the γ-secretase enzyme in postnatal skin, the IFE is hyperproliferative and hair follicles (HF) convert into cysts of cells undergoing IFE differentiation.[27] Conditional ablation of RBP-Jk gene results in a defect in the commitment switch between the basal to spinous cell fate, leading to a severely alterated differentiation program, with repressor of spinous and granular markers and loss of the spinous and granular layers, due to hypoproliferation of the epidermis, suggesting that Notch signaling is required for commitment of basal keratinocytes to suprabasal cell differentiation at early stages of epidermal development.[30] This result is in argument with previous studies[35,55] where an epidermal hyperproliferation was observed in mice with postnatal ablation of the Notch1 gene, suggesting that Notch1 signaling is required for basal keratinocytes to withdraw cell cycle and to promote terminal differentiation in postnatal epidermis. These contradictory roles of Notch1 signaling in keratinocyte proliferation and differentiation may be due to the involvement of either canonical or noncanonical Notch effects in the regulation of epidermal keratinocytes, or may reflect differences in the cell-context-specific function

of Notch signaling between embryonic and postnatal keratinocytes.[52] In a compromised epidermal barrier, epidermal hyperproliferation may be observed as a secondary response,[51] so it is also possible that the impaired epidermal differentiation suggested by conditional ablation of the RBP-Jk gene,[30] may provoke an indirect proliferative reaction in postnatal skin. Expression of a dominant negative form of Mastermind-like 1 causes hyperplastic and hyperkeratinized epidermis and epidermal cyst formation.[56] On the other hand, there are studies on the effects of activating the Notch pathway by overexpression of NICD in different epidermal layers. In the case of spinous layers of the IFE, such overexpression leads to expansion of the differentiated cell compartment, while when NICD is overexpressed in the basal layer of epidermis reduced expression of integrins, expansion of the IFE spinous cell layers and reduced granular differentiation are observed.[30,31,57]

Deletion of Notch receptors and ligands and the effects on epidermal homeostasis and differentiation were examined. As mentioned before, ablation of Notch1 gene causes significant alterations that are consistent with loss of normal growth control in epidermis and disruption of the well defined border between basal and upper differentiating layers, resulting in upregulation of integrin expression and epidermal hyperproliferation,[35,55] while deletion of Notch2, Notch3 or Notch4 alone does not have any reported effects on the epidermis.[27,58] Combined loss of Notch1 and Notch2 results in more severe epidermal phenotypes than loss of Notch1 alone by converting HF into cysts of cells undergoing IFE differentiation, suggesting that Notch2 also contributes to epidermal differentiation and combined deficiency of Notch1, Notch2 and Notch3 gives an IFE hypropoliferative phenotype.[27] Epidermal deletion of Jagged1 leads to conversation of HF into cysts of IFE, with thickening of the IFE, while Jagged2-deficient skin has no overt abnormalities in IFE.[31] Deletion of Dll1 in embryonic or adult epidermis results in increasing proliferation of the IFE keratinocytes and disturbed expression of the differentiation markers K10 and K17[40] suggesting that Dll1 contributes to the control of proliferation and differentiation in IFE. It has been reported that Dll1, which is expressed exclusively in basal layers, does not promote differentiation within the expressing cells when overexpressed. Dll1 maintains human epidermal stem cells in an undifferentiated state through homotypic interactions, but triggers commitment of neighboring Notch1-expressing keratinocytes to a transit amplyfing phenotype, through a negative feedback mechanism of lateral inhibition (noncell autonomous effect).[28]

In conclusion, different Notch ligands mediate different effects in vivo but also in vitro[31,40] in the expression of integrins and spinous markers, reflecting not only the different cell populations that express the ligands, but also the ability of the same cell population to respond differently to different ligands.[59]

CROSS-TALKS WITH OTHER PATHWAYS

During development, the establishment and stability of cell fates depend on the integration of multiple signals, which eventually modulate specific patterns of gene expression. In epidermal homeostasis and differentiation there are modulatory cross-regulatory interactions between Notch pathway and other signaling systems,[60] which are important to keratinocyte growth and differentiation (Fig. 1).

The levels of retinoic acid (Vitamin A) impact the terminal differentiation program in the suprabasal layers of multilayer epithelia, as low levels are associated with keratinized epithelia-like epidermis and high levels with nonkeratinized epithelia-like cornea. Notch1

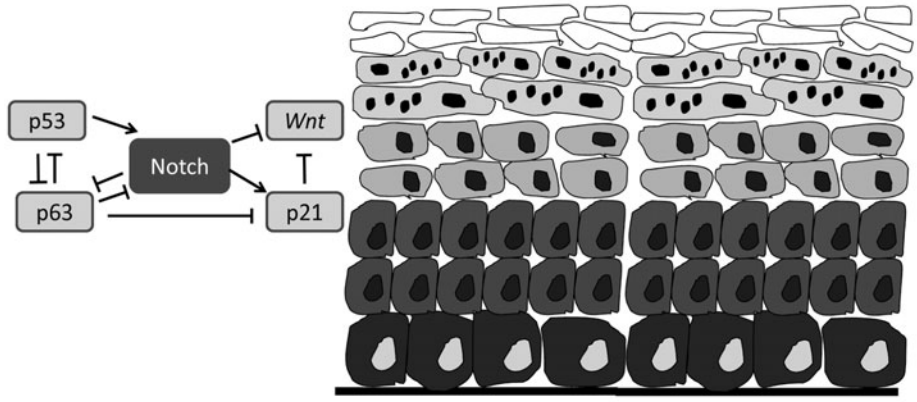


Figure 1. A complex network of Notch and other key signaling pathways regulates keratinocyte stem cell renewal, growth and differentiation.

signaling is linked to vitamin A metabolism by regulating the expression of the cellular retinol binding protein1 (CRBP1) which is needed for retinol metabolism in retinoic acid, suggesting an interaction between signaling pathways.[61]

The reciprocal crosstalking between Wnt and Notch pathway is very intriguing. Wnt signaling in embryogenesis is required to form HF and maintain them postnataly, while in adult epidermis its activation causes the conversion of HF into cysts of the IFE. The expression of several Notch pathway genes in epidermis is modulated by β-catenin , the key effector of Wnt pathway,[62] and Jagged1 expression on progenitor cells induced by canonical Wnt signaling activation leads to the maintenance of self-renewal potential of stem cells through Notch signaling activation.[63] Deletion of Jagged1 blocks β-catenin induced ectopic HF formation, without preventing the ability of β-catenin to stimulate differentiation. Whereas Notch activation (NICD overexpression) does not induce ectopic HF formation, it does promote differentiation within β-catenin induced follicles.[31] Similarly Notch activation may down-regulate Wnt pathway in two ways. By suppressing Wnts genes expression though the p21 key mediator, at the transcription-chromatin level,[64] or by binding of the Notch cytoplasmic domain to β-catenin, which negatively modulates β-catenin transcriptional activity.[65]

The oncogene c-Myc in human epidermal stem cells stimulates differentiation rather than uncontrolled proliferation and this seems to happen by a Myc-mediated down regulation of the cell adhesion genes, as reduced integrin-mediated adhesion is a positive terminal stimulus.[38] Indirect cross talk with Notch signaling can be hypothesized as p21 can bind to the N-terminus of c-Myc, suppressing c-Myc depended transcription[66] and c-Myc transcription can be activated by β-catenin.[67]

Nuclear factor κB (NF-κB) and peroxisome-proliferator activated receptor (PPAR) γ are two transcriptional regulators that are known to play key roles in keratinocyte differentiation and maturation. NF-κB activation plays a role in early stages of differentiation while PPAR activation participate in late differentiation events.[68,69] The Ank domain of Notch and an adjacent sequence bind to the p50 subunit of NF-κB and Notch has been proposed to alter the ratio of p50-p50 versus p65-p50 NF-κB dimmers, thereby affecting the selectivity of NF-κB-dependent transcription.[70] Nickoloff et al suggested a hypothesis

of cross-talk between Notch, NF-κB and PPARγ signaling in keratinocyte differentiation, where Jagged1 mediated activation of Notch signaling induce complete maturation of keratinocytes through NF-κB and PPARγ.[26]

p63, a member of the p53 family, plays a crucial role in the determination of keratinocyte cell fate, specifically in the transition between the simple and stratified epithelium of the epidermis and in the maintenance of the proliferative potential of basal keratinocytes.[10,71-73] Deletion of p63 gene promotes senescence and its increased expression suppresses differentiation.[72,74] Nguyen et al suggest that complex cross talk between Notch1 and p63 is involved in the balance between keratinocyte self-renewal and differentiation, where Notch1 activation down-modulates p63 expression, while p63 functions as a selective modulator of Notch1-dependent transcription and as an elevated expression of p63 counteracts the ability of Notch1 to restrict growth and promotes differentiation with antagonistic effects on Notch-responsive genes.[75] Data from a recent study suggest that the Notch downstream transcriptional factor Hes1 plays an important role in maintaining the p63 expression status and the proliferative potential of basal keratinocytes through a noncell-autonomous manner, in contrast to the alternative view that Notch signaling and p63 counteract each other in a cell-autonomous mechanism.[52]

After the discovery of AP-2 transcription factor binding sites in promoters of both basal and suprabasal epidermal genes, AP-2 proteins have been implicated in epidermal biology.[76,77] Mice lacking AP-2α and AP-2γ showed marked defects in terminal differentiation at the basal to spinous layer transition, resembling those caused by loss of RBP-Jk. Genetic studies[78] in *Drosophila melanogaster* proved a linear pathway between Notch and AP-2 expression and a possible functional link between Notch signaling, AP-2 transcription and induction of terminal differentiation in epidermal cells has been suggested.[79]

CONCLUSION

This report reviews the current knowledge of the role of the Notch signaling pathway in the control of epidermal differentiation and proliferation. Notch ligands and receptors are expressed in a dynamic pattern in the skin. The Notch signalling promotes differentiation of the hair follicle, sebaceous gland and interfollicular epidermal lineages and Notch acts as an epidermal tumour suppressor. Ablation of Notch1 gene causes significant alterations that are consistent with loss of normal growth control in epidermis and disruption of the well defined border between basal and upper layers, resulting in upregulation of integrin expression and epidermal hyperproliferation. To fulfil these functions, Notch signalling interacts with other pathways and acts via RBP-Jκ dependent and independent mechanisms. An important function of Notch signaling in the epidermis is the regulation of cell adhesion, as the location of a stem cell in particular sites within the epidermis determines the signals received from the local microenviroment which direct the cell to self-renew, or differentiation along distinct lineages. Current complexities of Notch and its many interactions with other signaling pathways, will evolve in the near future and will likely contribute to our better understanding of the role of the Notch signaling and the developing skin epidermis.

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CHAPTER 11

NOTCH SIGNALING AND THE DEVELOPING HAIR FOLLICLE

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Abstract: Notch function in the hair follicle has been mainly studied by use of transgenic mice carrying either loss or gain of function mutations in various members of the pathway. These studies revealed that whereas embryonic development of the hair follicle can be achieved without Notch, its postnatal development requires an intact Notch signaling in the hair bulb and the outer root sheath. Among the many roles played by Notch in the hair follicle, two can be highlighted: in the bulge, Notch controls a cell fate switch in hair follicle stem cells or their progenitors, preventing them from adopting an epidermal fate. In the hair bulb, Notch controls cell differentiation, ensuring the proper development of every layer of the hair shaft and inner root sheath. Notch function in the hair follicle is both cell autonomous and cell non autonomous and involves intercellular communication between adjacent layers.

INTRODUCTION

Hair follicles are the major appendages of the skin. They produce hairs involved in a number of functions including thermoregulation, collecting sensory information, protection against physical trauma and camouflage. Together with ist associated structures, namely the sebaceous gland, the apocrine gland and the arrector pili muscle, the hair follicle constitutes the pilosebaceous unit, a complex miniorgan of the skin. Hair follicle development occurs during embryogenesis but also throughout adult life as it is subjected to phases of growth and regression involving the cyclic recruitment of hair follicle stem cells. The development of hair follicles requires reciprocal signalings between epidermal

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and dermal compartments. In this chapter, the function of Notch signaling in the process of hair follicle development and renewal is reviewed.

DEVELOPMENT AND STRUCTURE OF THE HAIR FOLLICLE

The hair follicle has a mixed origin, as it contains cells deriving from both the epidermis and dermis. During embryogenesis, a series of reciprocal and coordinated signals between epithelial cells from the epidermis and mesenchymal cells from the dermis trigger the formation of hair follicles (Fig. 1).[1-4] Hair follicle development includes three main steps: hair follicle induction, leading to the formation of hair placodes which are small invaginations of epidermis into the underlying dermis; hair follicle organogenesis, the result of which is the hair peg; and hair follicle cytodifferentiation, leading to the formation of the bulbous peg.[4]

The initial cues determining the position and induction of hair placodes come from the dermis. As a result, epidermal cells proliferate as small downgrowths into the underlying dermis. The main factors involved in placode formation include Fibroblast Growth Factors (FGF), Bone Morphogenetic Protein (BMP)-inhibitory factors and Wingless (Wnt) signaling molecules. Placode growth requires additional signals coming from both dermal and epidermal cells within the placode. As it grows, the embryonic hair follicle further invaginates into the dermis. Epidermal cells signal to the mesenchyme through Sonic hedgehog to form a dermal aggregate, the future dermal papilla, that is surrounded by a group of mitotically active cells, the future matrix of the hair bulb (see Figs. 2,3). The matrix contains lineage-restricted precursor cells located around the dermal papilla. Reciprocal signals between the progenitor cells in the matrix and cells in the dermal papilla lead to activation of the differentiation program for the six layers of the fully grown hair follicle, creating concentric rings of differentiated cell types.[3-5] The proximodistal position of the progenitor cells within the hair matrix governs the fate of their progeny which moves upwards, exits the cell cycle and gradually expresses its differentiating program.[6]

The mature hair follicle undergoes phases of growth (anagen), regression (catagen) and quiescence (telogen) referred to as the hair cycle (Fig. 2). Not every part of the hair follicle is subject to morphological changes related to the phase of the cycle. Indeed, the upper third of the hair follicle does not cycle obviously and is thus known as the permanent portion, whereas the lower two-thirds, called the transitory portion, are subject to alternating phases of growth and regression depending on the phase of the cycle (Fig. 3).[7]

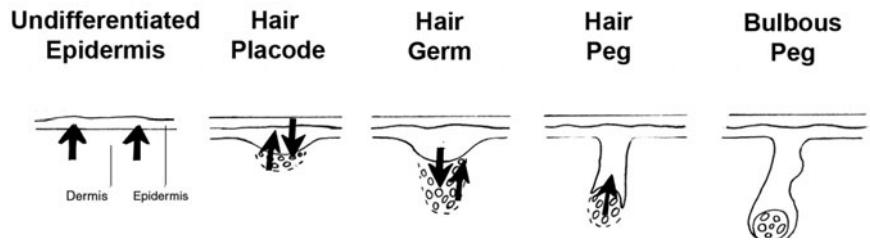


Figure 1. Embryonic development of a pelage hair follicle in the mouse. The sequential steps are indicated from left to right, with arrowheads representing the signals sent and received by the epidermal and dermal parts of the forming hair follicle. Adapted from Schneider MR et al. Curr Biol 2009; 19:R132-R142;[4] ©2009 with permission from Elsevier.

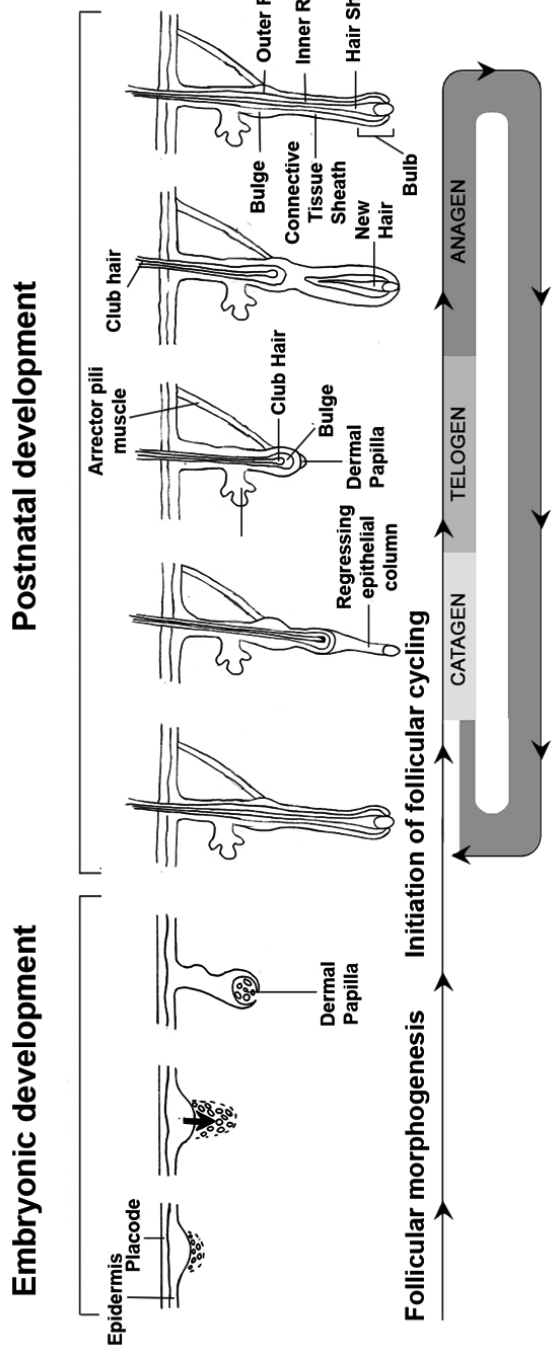


Figure 2. The hair cycle. Cycling starts just after completion of follicular morphogenesis and proceeds through phases of growth (anagen), regression (catagen) and quiescence (telogen). Adapted from Schneider MR et al. Curr Biol 2009; 19:R132-R142;[4] ©2009 with permission from Elsevier.

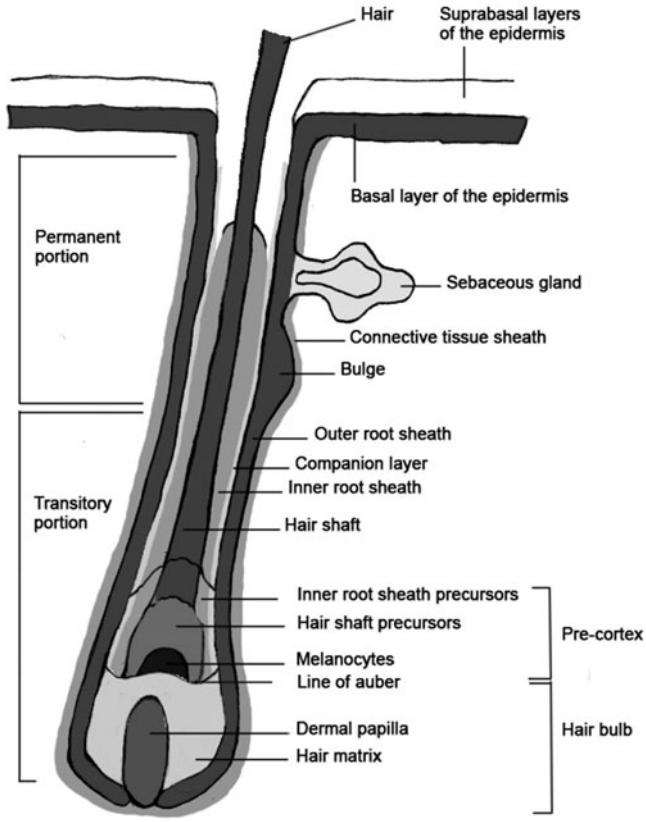


Figure 3. Structure of an anagen hair follicle in the mouse.

An anagen hair follicle is a highly organized, cylindric structure (Fig. 3).[7,8] At its center, the hair shaft consists of three layers of cells, the medulla, cortex and cuticle. It is surrounded by the inner root sheath (IRS), composed of the cuticle, Huxley and Henle layers. The hair shaft and IRS are surrounded by the outer root sheath (ORS) which is a stratified epithelium, continuous with the epidermis and separated from the IRS by the companion layer. They are produced by the mitotically active cells in the matrix of the hair bulb. The rapidly proliferating cells in the matrix are located under the "line of Auber", which separates them from the cells initiating differentiation along the lineages of the IRS and hair shaft. In consequence of cell differentiation, each cell layer above the line of Auber expresses specific genes (Fig. 4). The region above the line of Auber where cells differentiate is also known as the precortex. The matrix also contains melanocytes, lying against the dermal papilla and responsible for synthesis and transport of melanin granules towards the keratinocytes of the forming hair. In the uppermost part of its permanent portion, the hair follicle is surrounded by the sebaceous gland which secretes lipids into the hair canal. Just under the sebaceous gland, the ORS presents a convex extension known as the bulge where the stem cells for keratinocytes and melanocytes are found.

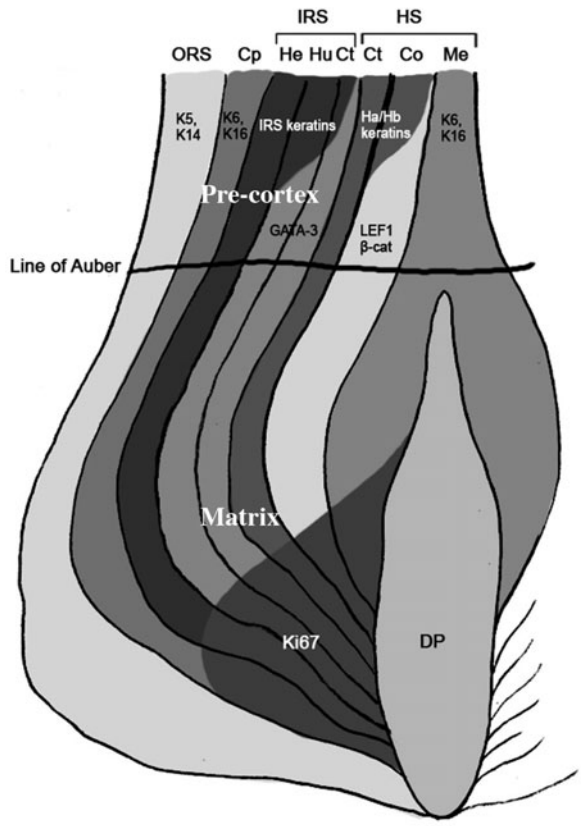


Figure 4. Genetic markers for the differented lineages in the hair follicle. The line of Auber delimits the matrix containing proliferative, Ki67 expressing cells and the precortex where cells start differentiating and expressing specific markers : Keratin 5 (K5) and Keratin 14 (K14) in the outer root sheath (ORS) ; Keratin 6 (K6) and keratin 16 (K16) in the companion layer (Cp) ; inner root sheath (IRS) keratins in the three IRS layers : Henle layer (He), Huxley layer (Hu) and cuticle (Ct) and GATA-3 in He and Ct ; hair acidic and basic (Ha/Hb) keratins in the cuticle (Ct) of the hair shaft (HS) ; Lymphoid enhancer binding factor 1 (LEF1) and β-catenin (β-cat) in the HS cortex ; K6 and K16 in the HS medulla. DP: dermal papilla. Adapted with permission from: Fuchs E. Nature 2007; 445(7130):834-42;[1] ©2007 Macmillan Publishers Ltd.

During catagen, the matrix cells stop proliferating and the melanocytes cease to produce pigment. Both cell types undergo massive apoptosis, leading to the degeneration and shortening of the hair follicle. The connective tissue surrounding the degenerating hair follicle shrinks in such a way that the dermal papilla is moved upwards. During catagen, a specialized structure called the club hair is formed by the ORS cells, anchoring the hair shaft to the follicle and allowing it to be retained through several subsequent hair cycles (Fig. 2).

During telogen, the hair follicle reaches its shortest size so that the dermal papilla is in contact with the hair bulge (Fig. 2). It allows exchange of signals between the dermal papilla and the hair bulge to occur, mimicking those occuring during embryogenesis, stimulating the stem cells and leading to the beginning of a new anagen.

EXPRESSION OF MEMBERS OF THE NOTCH PATHWAY IN THE HAIR FOLLICLE

Expression of members of the Notch pathway in the hair follicle has been studied in the mouse by In Situ Hybridization,[9-17] immunofluorescence microscopy and immunohistochemistry,[10,17-24] by use of transgenic mice carrying a Green Fluorescent Protein reporter for Notch activity (TNR mice),[19] a β-galactosidase reporter gene,[25] or a genetic sensor of Notch1 proteolysis in vivo (N1IP-CRE mice).[26] The expression pattern for several but not all receptors, ligands and target genes has been studied and is summarized below.

Expression during Embryogenesis

During embryogenesis, *Notch1* mRNAs are found in the epidermal part of the hair placode, mostly the inner cells of the epidermal invagination, but not in its mesenchymal part.[13,14] At the bulbous peg stage of development (see Fig. 1), *Notch1* is expressed both at the mRNA[14] and protein[10] level in the hair bulb and in internal cells, but not in presumptive ORS cells.

Notch2 is not expressed in embryonic hair follicles.[12]

Delta1 is expressed during embryogenesis only, in the mesenchymal cells of the presumptive dermal papilla.[14]

Jagged1 and *2* transcripts are found in the same locations as *Notch1* transcripts, that is in the epidermis and the epidermal parts of the forming hair follicle.[14]

Delta like 1 is expressed in the dermal papilla and the bulb of embryonic hair follicles, but not in adult hair follicles.[25]

Hes1 protein is found in the same locations as Notch1 in the hair peg at embryonic day 17 (E17): in the suprabasal core of cells forming the presumptive IRS. At P0, when the hair follicle matures, Hes1 is also expressed in the hair shaft.[10] At E16.5, *Hes1* is highly expressed in melanoblasts which also contain low amounts of *Hes5* and *Hey1* transcripts.[21]

Lunatic-fringe, *Manic-fringe* and *Radical-fringe*, encoding three secreted proteins modulating Notch signaling, are expressed in the hair follicle of the vibrissa at embryonic day 18.5 (E18.5).[12,15]

All these data suggest that there are at least two pairs of proteins involved in deciding where hair placodes will be formed: Notch2 and Delta 1 in the dermis and Notch1 and Serrate 2 in the epidermis. Notch2, Delta1 and Lunatic Fringe genes may be required for dermal papilla formation, whereas Notch1, Serrate2 and Lunatic Fringe may be implicated in the patterning of the epidermal cells.[12]

Expression during Postnatal Life

In adult anagen hair follicles, *Notch1* transcripts are found in the inner cells of the hair bulb and in the suprabasal cells of the ORS.[14] Notch1 intracellular domain (NICD), which is produced after Notch1 activation, is strongly expressed in undifferentiated hair matrix cells and cells of the cortex and cuticle of the hair shaft; it is also expressed in a few cells in the ORS and in the cuticle layer of the IRS.[23,17] In anagen hair follicles, Notch1 activity is detected above the line of Auber in the upper part of the hair bulb where cells undergo differentiation, in the cuticle of the IRS, in the ORS and in the dermal papilla.[19]

In adult hair follicles, subpopulations of cells with Notch1 activity are infrequently found in the bulge region where both keratinocyte and melanocyte stem cells reside.[26]

Notch2 transcripts are found in the IRS precursors, distal to *Notch1* and in the differentiated IRS cells.[23]

Notch3 protein is expressed distally to Notch1 in the cortex of the hair shaft and in the sebaceous gland.[23]

The expression of *Delta1* is not detected in adult hair follicles.[14]

Jagged1 is expressed in the matrix, in precortex cells destined to form the hair shaft cortex and cuticle and in the suprabasal cells of the ORS above the bulb.[9,14,19] Jagged1 expression and Notch activity seem to be elevated in regions of the epidermis where cells undergo commitment to terminal differentiation and thus are associated with transit amplifying cells rather than stem cells.[19]

Jagged2 expression is mainly restricted to the bulb cells next to the dermal papilla and in the basal cells of the ORS.

Jagged1, *Jagged2* and *Notch1* are not expressed in advanced, differentiating hair shaft or IRS cells.[14]

RBPJk is expressed in the anagen hair follicle, including the bulge region.[24]

Hey1 protein is found in cortex and cuticle precursor cells of the hair shaft.[10]

Hes1 protein is mostly expressed in the IRS;[10] its mRNA is abundant in the matrix and the precortex, is widely detected in the cortex, medulla and cuticle of the hair shaft and is expressed in a few ORS cells.[9]

Hey2 is expressed in dermal papilla cells and *HeyL* in the IRS and hair shaft, with some expression in the precortex. *Hes5* transcripts are found in the differentiating hair shaft medulla and in small, asymmetrical patches in the bulb near the line of Auber, in cells that may belong to the melanocyte lineage.[9] Melanocytes express NICD, Hes1 and HeyL proteins, the three corresponding genes being overexpressed in melanocyte stem cells within the bulge compared to melanocytes in the bulb.[27]

Expression of Notch target genes in the precortex reflects activation of canonical Notch pathway as matrix cells get commited to terminal differentiation to form the hair shaft and the IRS.[9]

Figure 5 gives a summary of expression patterns of members of the Notch pathway in anagen hair follicles.

Telogen hair follicles express *Jagged1* and *Hes1* at low levels, but not *Hes5*, *Hey2*, *HeyL* or *Hey1*.[9]

Expression of ligands, receptors and target genes of the Notch pathway in both embryonic and postnatal hair follicles suggests a role for that pathway in the development of hair follicles during embryogenesis and postnatal life.

NO ESSENTIAL ROLE FOR NOTCH IN THE INITIAL STEPS OF EMBRYONIC HAIR FOLLICLE DEVELOPMENT

The role of Notch pathway in hair follicle development has been mainly studied by generating mouse mutants carrying either loss of function or gain of function mutations in members of the pathway that were known to be expressed in the hair follicle.[9,10,18-21,23-25,28-31] As the invalidation of many genes of the Notch pathway is embryonic lethal, conditional or inducible mutants were producedj in which the targeted gene is deleted in a specific cell type or at a chosen moment. It can be achieved for instance by use of the Cre/loxP

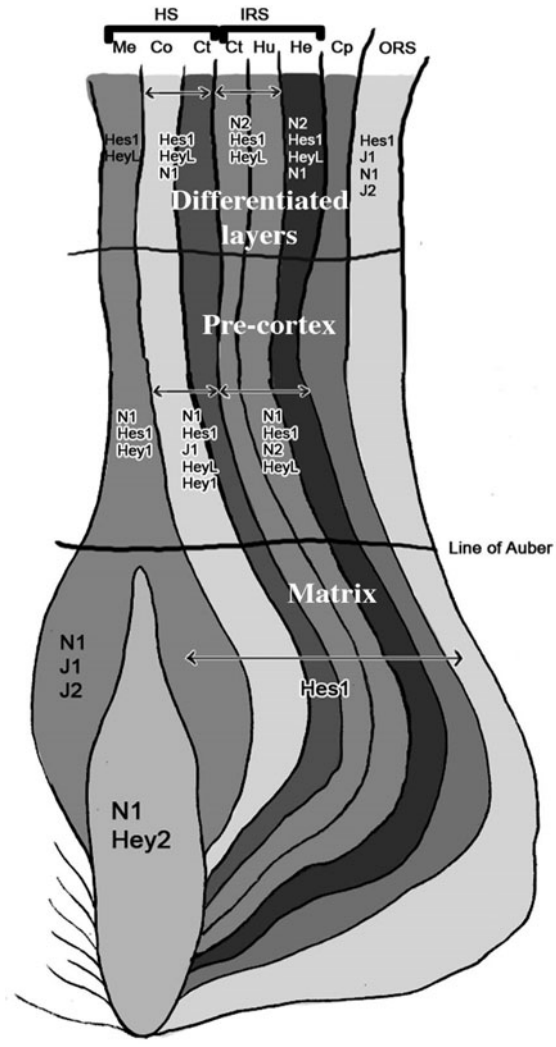


Figure 5. Expression patterns of members of the Notch pathway in an anagen hair follicle in the mouse. N1: Notch1; N2: Notch2; J1: Jagged1; J2: Jagged2; HS: hair shaft; IRS: inner root sheath; Me: medulla; Co: cortex; Ct: cuticle: Hu: Huxley layer; He: Henle layer; ORS: outer root sheath.

system for cell-specific invalidation, jointly with the tetracycline-regulated transcriptional system for inducible invalidation (see refs. 32, 33 for a review). Table 1 gives a summary of the various mutants cited in this chapter.

In every conditional knock-out for a gene in the Notch pathway in hair follicles produced so far, the number and distribution of hair follicles in the skin of newborn mutant mice are comparable to those of wild-type mice.[10,18-20,23-25,34] Accordingly, in mutant mice deleted for *Notch1* in the basal epidermis the periodicity, spacing and the number of placodes are not altered.[34] Moreover, expression patterns of signaling molecules of the

Table 1. Summary of the mutant mice cited in the chapter. LOF: loss of function mutation; GOF : gain of function mutation

Mutation Types	Nature	Transgenes Symbol Used in the Manuscript	Expression Pattern in Adults	Refs.
Notchl conditional and inducible LOF	*K5-Cre^{ERT}; Notch1$^{flox/flox}$*	K5N1cKO	ORS and basal layers of the sebaceous gland	34
Notchl conditional LOF	*K14-Cre; Notch$^{flox/flox}$*	-	ORS and basal layers of the sebaceous gland	34
Notchl GOF	*MHKA1-Notch1$^{\Delta E}$*	-	Hair cortex	29
Notchl GOF	*Ivl-Notch1lC*	-	IRS	31
Notch1 inducible GOF	*K14-N$^{ICD\Delta OP}$ER*	-	ORS and basal layers of the sebaceous gland	19
RBPJk conditional LOF	*K14-Cre; Rbpjk$^{flox/flox}$*	K14RBPJcKO	ORS and basal layers of the sebaceous gland	14
Jaggedl conditional LOF	*K5-Cre*; *Jag1$^{flox/flox}$*	K5J1cKO	ORS and basal layers of the sebaceous gland	19
Notchl conditional LOF	*Msx2-Cre; Notch1$^{flox/flox}$*	Msx2N1cKO	Hair matrix	23
Notch2 conditional LOF	*Msx2-Cre; Notch2$^{flox/flox}$*	Msx2N2cKO	Hair matrix	23
Notchl and 2 conditional LOF	*Msx2-Cre; Notch1$^{flox/flox}$; Nctch2$^{flox/flox}$*	Msx2N1N2cKO	Hair matrix	23
Notch3 LOF	*Notch3$^{-/-}$*	N3KO	All Notch3 expressing cells	23
Notchl and 2 conditional LOF and Notch3 LOF	*Msx2-Cre; Natch1$^{flox/flox}$; Notch2$^{flox/flox}$; Notch3$^{-/-}$*	Msx2N1N2cKON3KO	Hair matrix	23
RBPJk conditional LOF	*Msx2-Cre; Rbpjk$^{flox/flox}$*	Msx2RBPJcKO	Hair matrix	18
RBPJk conditional LOF	*Nestin-Cre; Rbpjk$^{flox/flox}$*	NesRBPJcKO	Hair follicle stem cells	24
RBPJk conditional LOF	*K15-Cre; Rbpjk$^{flox/flox}$*	K15RBPJcKO	Hair follicle stem cells	18
Notchl and 2 conditional LOF and Notch3 LOF	*K15-Cre; Notch1$^{flox/flox}$; Nctch2$^{flox/flox}$; Notch3$^{-/-}$*	K15N1N2cKON3KO	Hair follicle stem cells	18
Delta like 1 conditional LOF	*K5-Cre; Dll1$^{flox/flox}$*	K5Dll1cKO	ORS and basal layers of the sebaceous gland	25
RBPJk conditional LOF	*Tyr-Cre; Rbpjk$^{flox/flox}$*	TyrRBPJcKO	Melanocyte lineage	21,43
Notchl and 2 conditional LOF	*Tyr-Cre;Notch1$^{flox/flox}$; Nctch2$^{flox/flox}$*	TyrN1N2cKO	Melanocyte lineage	30

Tumor Necrosis Factor, Bone Morphogenetic Protein and Sonic hedgehog families known to be involved in early placode formation and/or in embryonic hair follicle development are not altered in the mice with a *Notch1* loss of function mutation.[34] Thus the Notch pathway is not essential for embryonic patterning or initial specification/differentiation of hair follicles. This is in contrast to the embryonic development of feathers, where Notch plays a role in promoting feather bud development and patterning [35] and in establishing the anterior-posterior asymmetry in feather buds.[36]

However, deletion of *Notch1* in Keratin 14 expressing cells, which starts at E14.5 in the skin, is associated with an increase in hair follicle number in the dermis at E17.5 compared to littermate controls, even if at postnatal day 2 (P2), hair follicle invagination and number are normal in the mutants.[34] It suggests that the placode cells invade the dermis earlier in absence of Notch1 signaling and thus that Notch1 is involved in regulating the onset of hair follicle invagination during embryogenesis.

NOTCH AND THE MAINTENANCE OF CELLULAR IDENTITY WITHIN THE HAIR FOLLICLE

Whereas Notch is not necessary for initial development of hair follicles, it plays a key role in cell differentiation within the hair follicles during late embryogenesis and adult life.

Gain of Function Mutations in Notch Pathway Act Non Cell Autonomously on Hair Follicle Differentiation

The first report of such a role for Notch describes the overexpression of an active form of Notch1 (*Notch1*$^{\Delta E}$) in precursor- and differentiated cells of the hair cortex in *mouse hair keratin A1 MHKA1-Notch1*$^{\Delta E}$ transgenic mice.[29] The transgenic mice express the active form of Notch1 under the control of the *MHKA1* promoter. They display curly whiskers and a shiny, wavy fur from the first anagen of the hair cycle, at P9. They start losing hairs around P40, at the transition between catagen and telogen during the second hair cycle and at the same stage in all the following hair cycles. In the transgenics, morphology of the IRS and ORS, hair growth and expression of IRS and hair shaft medulla markers are normal. In the upper half of the transgenic hair follicles, the cuticle is disorganized and the medulla does not contain air so that the curvature and refractory properties of the hair shaft are impaired. As *MHKA1* is not expressed in either the medulla or the cuticle, it suggests that overexpression of activated Notch1 in the hair cortex precursor- and differentiated cells affects the differentiation program in the medulla and cuticle. Altered cellular interactions in the cortex may induce the expression of a secreted or surface protein, capable of altering the differentiation program in its two adjacent cell layers, the cuticle and medulla.[29]

Such a non autonomous function for Notch signaling has also been found in transgenic mice overexpressing a constitutively activated Notch1 in involucrin expressing cells (*Ivl-Notch1IC* mice).[31] In these mice, the involucrin promoter drives the expression of activated *Notch1* in the suprabasal layer of the interfollicular epidermis and in the IRS. In the transgenics, appearance of the first coat is delayed and the hairs are short, wavy, dull and randomly orientated. A peculiar alopecia phenotype develops from P25 as it initially preserves the midline hairs, conferring a “Mohawk-like” appearance

to the mice. The mice are completely bald by P30 and they regrow their coat with a similar pattern in the subsequent hair cycles. In the IRS of transgenics, the cells of Henle's and Huxley's layers fail to mature and show a delay in keratinization so that no fully hardened IRS is produced. The defects in IRS differentiation include retention of nuclei within the upper portion of the IRS and the continuation of the IRS above the level of the sebaceous gland. The failure in differentiation and keratinization is more pronounced in the Henle's layer than in the Huxley's layer. This suggests that Notch signaling in IRS precursor cells controls the development of the different cell types within the IRS. However, Notch plays also a role in the differentiation of adjacent layers in the hair follicle. Indeed, the hair shafts of the *Ivl-Notch1IC* mice are dystrophic and display abnormalities in their three layers. Moreover, the cells of the ORS in contact with the IRS have an abnormal morphology and the ORS fails to produce club hairs. As the involucrin promoter is exclusively expressed in the IRS of the hair follicles, it is hypothesized that the defects in the hair shaft and ORS are a consequence of the abnormalities described in the IRS which makes it unable to interact with its neighbouring layers.

Alopecia may also occur when activated Notch1 is expressed in ORS cells. Indeed, overexpression of an activated form of Notch1 in Keratin 14 positive cells, including the ORS and the periphery of the sebaceous gland, has been obtained through an inducible system ($K14N^{ICD\Delta OP}ER$ mice).[19] In the newborn mutants, the hair follicles are clumped and the hairs are not uniformly oriented. In 13 weeks mutants, the hair bulbs are enlarged and lose contact with the rest of the follicles, forming cysts consisting of an enlarged bulb surrounding an aberrant hair shaft. The cysts express markers of the companion layer, the hair bulb and the IRS, but also of terminal differentiated interfollicular epidermis. Altogether, overexpression of activated Notch1 in the ORS alters the differentiation program not only of the ORS but of several other layers in the hair follicle.

In those three models, alteration of Notch signaling was artificially obtained through either over- or ectopic expression of activated Notch1 in different layers of the hair follicle. To gain insight into the role of Notch signaling under normal conditions, several loss of function mutations were produced which ablate the signaling in hair follicle cells known to express the gene.

Loss of Notch Function in Hair Follicles Leads to Alopecia and/or Alteration in the Differentiation Program

Several loss of function mutations for members of the Notch pathway were produced in ORS cells by driving Cre expression either in Keratin 14 (K14) or K5 expressing cells.[10,19,34] Keratin 5 and 14 start being expressed at E9.25 and E9.75 respectively in the basal layer of the ectoderm.[37] In adult hair follicles, both K14 and K5 are expressed in the ORS and the basal layers of the sebaceous gland.[19]

In mice invalidated for *RBPJk* in K14 expressing cells (*K14–Cre*/+; $RBPJk^{flox/flox}$ (K14RBPJcKO) mice), the hair follicles are smaller and show an impaired differentiation at birth.[10] In the IRS of K14RBPJcKO hair follicles at birth, fewer cells express the GATA-3 marker, specific for the IRS and the cells that express GATA-3 do so at a lower level than in wild-type hair follicles. This suggests that Notch/RBPJk signaling is required during the earliest stages of IRS differentiation. Later stages of hair follicle differentiation are also impaired: in K14RBPJcKO hair follicles in late anagen, few hair channels are formed; the remaining air channels are filled with keratins instead of air; no

hair shaft or IRS structures can be identified. The hair follicles of K14RBPJcKO mice eventually degenerate into cystic structures.

Similarly, the loss of *Notch1* function in the Keratin 14 positive cells of *K14–Cre*/+; *Notch1*$^{flox/flox}$ (K14N1cKO) mice is associated with a pronounced hair loss and a dramatic reduction in the number of hair follicles in adults.[34] At P13, the remaining hairs are thinner, shorter and wavy, with an aberrant structure and the scales in the cuticle are irregular and broken. The bulb regions of the remaining hair follicles are involuted.

In mice invalidated for *Jagged1* in Keratin 5 positive cells (*K5–Cre*/+; *Jag1*$^{flox/flox}$ (K5J1cKO) mice),[19] invalidation starts at E15.5 in the basal layer of the epidermis. The K5J1cKO mice cannot be distinguished from controls at birth but start losing their hairs and whiskers at 5 weeks and become eventually bald by 7.5 weeks, with only a few sparse, short and thin hairs left. This phenotype is very similar to what is seen in *Notch1* conditional knock-out mice, suggesting that *Jagged1* is the major ligand for Notch signaling in postnatal skin.

In conclusion, Notch/RBPJk signaling in the ORS plays a role in IRS/hair shaft differentiation from matrix cells.

Further insight into Notch1 function in hair follicle differentiation was gained by invalidating *Notch1* in the Msx2 expressing cells of *Msx2–Cre*/+; *Notch1*$^{flox/flox}$ (Msx2N1cKO) mice.[23] In this system, *Cre* expression starts at E9.5 in the basal layer of the ectoderm and a second wave of *Cre* expression is initiated in the matrix of the hair follicles at P1. In the Msx2N1cKO mice at birth, the hairs have an altered morphology. They are wavy, finer and shorter than in controls and display bulbous alterations in the shaft. Every follicular cell type is present in the Msx2N1Msx2cKO hair follicles, but the medulla of the hair shaft and the Henle layer of the IRS are disorganized. Moreover, the expression of the medulla marker Keratin 17 is drastically reduced in the medulla of Msx2N1Msx2cKO hair follicles. Since under the line of Auber Notch1 protein is expressed in the proximal cells of the hair matrix but that above the line of Auber its expression is restricted to cortical and cuticle cells, the action of Notch1 on medulla differentiation has to be non cell autonomous.[23]

Although *Notch2* and *Notch3* are expressed in the hair follicle, the invalidation of either *Notch2* in the basal layer of the ectoderm in *Msx2–Cre*/+; *Notch2*$^{flox/flox}$ (Msx2N2cKO) or in *Notch3*$^{-/-}$ (N3KO) mice does not lead to any obvious skin phenotype.[23] However, when both *Notch1* and *Notch2* are invalidated in the basal layer of the ectoderm (Msx2N1N2cKO), the double mutants display a worse phenotype than the Msx2N1cKO single mutants, revealing some redundancy between Notch1 and Notch2 in the hair follicle. The Msx2N1N2cKO newborn have patches of naked skin that become scaly and thick. The naked regions contain hair follicles with an abnormal structure: whereas the IRS cells are initially formed, by P8, the IRS cannot be identified and is replaced by a core of pigmented, loosely packed cells, forming a degenerated hair shaft. The Msx2N1N2cKO mice die around P25.[23]

The Msx2N1N2cKO phenotype is comparable to what is seen when γ-secretase is invalidated in the basal layer of the ectoderm, preventing all four Notch receptors to be activated.[23] This suggests that Notch1 and 2 are the only Notch receptors involved in hair follicle differentiation. In support to this view, removing *Notch3* in an Msx2N1N2cKO background does not aggravate the hair follicle phenotype seen in Msx2N1N2cKO mice.[23]

It is interesting to mention that a role for noncanonical Notch signaling in hair follicle differentiation has been reported. Indeed, mice invalidated for *RBPJk* in Msx2 expressing cells (Msx2RBPJcKO mice) have a milder hair follicle phenotype than mice invalidated

for *Notch1* and *Notch2* in Msx2 expressing cells (Msx2N1N2cKO). Furthermore, the Msx2N1N2cKO mice display the same phenotype as mice invalidated for *Notch1*, *Notch2* and *RBPJk* in Msx2 expressing cells (Msx2N1N2RBPJcKO).[18]

Altogether, these data show that Notch is necessary for maintaining the differentiated cellular identity of the IRS, hair shaft and ORS and that its function in hair follicle differentiation is entirely mediated through Notch1 and Notch2. Notch1 and 2 act autonomously in IRS whereas Notch1 acts non-autonomously in the hair shaft.

As similar phenotypes are observed in loss- and gain of function mutants for Notch, the level of Notch signaling must be important for normal hair follicle differentiation.

NOTCH AND THE REGULATION OF CELL FATE IN HAIR FOLLICLES

One of the well-known functions for the Notch signaling pathway during mouse development is control in cell fate decisions. Such a role is fulfilled in the hair follicle. Indeed, several conditional loss of function mutations in members of the Notch pathway lead to a switch of fate in ORS cells that adopt an epidermal program. In consequence the hair follicles are replaced by epidermal cysts.

K5J1cKO mice, invalidated for *Jagged1* in K5 expressing cells and K14N$^{ICD\Delta OP}$ER mice overexpressing activated Notch1 in K14 expressing cells do develop epidermal cysts.[19] The cysts from K5J1cKO mice are filled with cornified material. They express Keratin 10, a marker specific for interfollicular epidermis, but not markers of the IRS. On the contrary, the cysts from K14N$^{ICD\Delta OP}$ER mice are expansions of the hair follicle, they resemble massive hair bulbs with aberrant hair shafts and they express several IRS markers. Whereas cysts can be the consequence of both loss and gain of Notch function, they seem to be linked to different events: a switch toward epidermal cell fate for the loss of function mutation and an aberrant differentiation for the gain of function mutation.

The study on K14N1cKO mice, invalidated for *Notch1* in K14 expressing cells, supports the aberrant differentiation hypothesis. When K14N1cKO mice reach 4 weeks of age, their hair follicles are either lost or transformed into small cysts. The cysts contain a multilayered epithelium expressing Keratin 1 (K1), an early differentiation marker for interfollicular keratinocytes.[34] They do not express Loricrin, a late differentiation marker for interfollicular epidermis. The authors of this study interpret cysts formation not as an impaired choice in cell fate between hair and interfollicular skin in hair follicle precursor cells. They rather think that without Notch1, the hair follicle differentiation program is altered, leading to epidermal cyst formation by default.

In Msx2N1N2cKO mice that are invalidated for both *Notch1* and *Notch2* in Msx2 expressing cells, initiation of catagen triggers stratification and keratinization of the ORS which eventually converts into epidermal cysts.[23] In wild-type skin, ORS cells can contribute to the interfollicular epidermis only after skin injury but not under normal conditions. Thus, in wild-type mice, either the ORS cells respond to a stimulating signal produced by the wound when the skin is injured and convert into epidermal cells, or catagen hair follicles produce an inhibitory signal preventing the ORS cells to do so in the absence of skin injury. In Msx2N1N2cKO mice, the hair follicles either produce the stimulatory signal or fail to produce the inhibitory one. Whatever the reason, in absence of Notch1 and Notch2 signaling, there is a change of destiny in ORS cells which adopt an epidermal program during catagen.[23]

In these systems leading to Notch deficiency in hair follicles, the switch from a hair follicle to an epidermal differentiation program and cyst formation may have two origins: a change in cell fate in keratinocyte stem cells, or a defect in the differentiation of committed hair follicle progenitors. Whether the change of destiny occurs through a cell fate switch in bulge stem cells or through an impaired differentiation in their progenitors is not discussed in this study but the question is addressed in the two following ones:[18,24]

Mice invalidated for *RBPJk* in nestin expressing cells (*nestin–Cre*/+; $RBPJk^{flox/flox}$ (NesRBPJcKO) mice) have a normal coat at birth but their skin displays hair loss, epidermal hyperkeratinization and cyst formation when the mice reach 1 to 3 months of age.[24] The cysts express markers for all stages of epidermal differentiation, including the undifferentiation marker K5, the early differentiation markers K1 and Involucrin and the terminal differentiation markers Filaggrin and Loricrin. Differentiation within the cysts proceeds from the basal to the apical layer, with the few basal layers being highly proliferative. Hair follicle stem cells in the bulge are nestin positive and contribute to both epidermis and hair. Their progenitors destined to become epidermal cells move upwards in the upper permanent portion whereas the progenitors destined to become hair follicle cells move downwards toward the hair bulb. Tracing experiments revealed that in the hair follicles of NesRBPJcKO mice, more progenitors of the nestin positive hair follicle stem cells are found above the bulge in the upper permanent portion than in control mice. These cells elect an epidermal cell fate at the expense of the hair follicle cell fate and contribute to cyst formation. The authors of this study hypothesize that Notch/RBPJk signaling may negatively regulate the epidermal cell fate in hair follicle stem cells and facilitate their differentiation into hair cells.[24]

Finally, transgenic mice were produced in which canonical Notch signaling was removed specifically in bulge stem cells (K15RBPJcKO mice).[18] In these mice, deletion of *RBPJk* is accompanied by expression of a *lacZ* reporter gene in the deleted stem cells and their progeny, allowing cell fate mapping experiments. As expected, the K15RBPJcKO mice develop aberrant hair shafts and epidermal cysts. In these mice, β-galactosidase expressing cells are found in the hair matrix, the cortex and the cuticle of the hair shafts, proving that canonical Notch signaling is not required for hair follicle fate selection in descendents of keratinocyte stem cells. When loss of Notch1 and Notch2 function in K15 cells is combined with loss of Notch3 function (K15N1N2cKON3KO mice), Notch-deficient stem cells also give progeny contributing to hair follicle structures.[18] Hence, neither canonical nor RBPJk-independent Notch signaling is required for stem cells to choose the follicular fate.

In both K15RBPJcKO and K15N1N1cKON3KO mice, a significant number of progeny of the deleted stem cells migrate upward from the bulge to the isthmus and epidermis where they express markers of interfollicular epidermis.[18] The upward migration toward the epidermis starts before the follicles enter anagen and thus precedes the formation of aberrant hair shafts and cysts, demonstrating that improper differentiation is a consequence of the fate switch. In control mice, no cells migrate from the bulge toward the epidermis. In conclusion, a functional Notch signaling is required in hair follicle stem cells or their uncommitted descendents to prevent them from adopting the epidermal fate which, by default, restricts them to the follicular fate.

ADDITIONAL ROLES FOR NOTCH IN HAIR FOLLICLES

Control of Hair Follicle Cycling

Transient overexpression of activated β-catenin in K14 expressing ORS cells induces telogen hair follicles to enter the hair growth cycle. This shows that β-catenin alone is able to induce anagen.[38] This is not the case when an activated form of Notch1 is overexpressed also in K14 expressing cells (K14N$^{ICD\Delta OP}$ER transgenics); in this case, telogen hair follicles stay in the resting phase.[19] Thus activation of Notch alone is not sufficient to induce anagen.

Similarly, the deletion of *Notch1* in Msx2 expressing cells (Msx2N1cKO) during embryogenesis leads to shorter hair follicles the bulbs of which contain less cells than control bulbs.[20] Such a phenotype might be due to an accelerated catagen onset. However, observation of hair follicle structure in Msx2N1cKO mice from P9 to P22 did not reveal any change in the timing of catagen or telogen onset.

Nevertheless, several lines of evidence show that Notch is involved in controlling hair follicle cycling:

In mice invalidated for *Delta like 1* in K5 expressing cells (K5Dll1cKO), there is a delayed entry into the first postnatal anagen which occurs at 5 weeks in controls.[25] However, subsequent hair cycles in mutants proceed at the same pace as in controls. No other defect is observed in the hair follicles of K5Dll1cKO mice.

In transgenic mice expressing the active form of Notch1 under the control of the *MHKA1* promoter, the third hair cycle starts earlier (P57) than in wild-types (P89), suggesting a role for Notch in controlling the cycle clock.[29]

Such a role for Notch has been confirmed in mice invalidated for *Notch1* function in K14 or K5 expressing cells: at P9, wild-type hair follicles are in anagen and the matrix cells are actively proliferating, whereas very few proliferating cells are found in the hair matrix of *Notch1* deficient mice.[34] Such a low proliferative rate is typical of the end of anagen. The lack of proliferation is not linked to an increase in apoptosis in the mutant hair matrix. In conclusion, without Notch signaling, hair follicles enter catagen prematurely. The mechanism by which Notch controls catagen entry is not known.

Control of Cell Proliferation and Apoptosis in Hair Bulbs

The hair follicles in mice invalidated for *Notch1* in Msx2 expressing cells (Msx2N1cKO) during embryogenesis have smaller bulbs, containing a reduced number of cells compared to controls.[20] In the mutant matrix cells at P9, mitotic rates are severely reduced, as revealed by bromodeoxyuridine (BrdU) and Ki67 labellings and apoptotic rates are slightly elevated. To investigate this phenotype, microarray analyses were performed on Msx2N1cKO hair bulbs. They revealed an elevated expression of cytostatic genes such as Cyclin kinase inhibitors *Cdkn1a*, *2b* and *2c*, encoding respectively p21^{Cip1}, p15^{INK4B} and p57^{Kip2} and of some proapoptotic genes such as *Scotin*.[20]

Interestingly, the expression of several Insulin Growth Factor 1 (IGF1) signaling modulators including *Igfbp2*, *3* and *4* from the Insulin Growth Factor Binding Protein (IGFBP) family and of IGF receptor are elevated in Notch1-deficient hair bulbs.[20] IGF1 signaling has mitogenic and anti-apoptotic activities which are antagonized by the IGFBP through their IGF1 sequestrating activity.[39] In transgenic mice ectopically expressing IGFBP3 in the involucrin expressing IRS and medulla (*Ivl-Igfbp3* mice), the hair bulbs are

shrunk and the structure of the hair shafts is altered[40] whereas overexpression of IGF1 in the IRS and medulla of *Ivl-Igf1* mice triggers proliferation in the matrix.[41] These data show that IGF1::IGFBP ratio controls cell proliferation in the matrix. Combining loss of *Notch1* function in Msx2 expressing cells and gain of *Igf1* function in Involucrin expressing cells (Msx2N1cKO; *Ivl-Igf1* mice) rescues the proliferation defect of Notch1-deficient matrix cells.[20] Hence, the hair bulb phenotype in Msx2N1cKO Notch1 deficient mice is due to an imbalance between IGF1 and IGFBP expression. In hair bulbs, Notch1 and IGFBP2 are expressed in matrix cells whereas IGF1 and IGFBP3 are exclusively expressed in fibroblasts of the dermal papilla.[20,41] Thus in Msx2N1cKO mice, change in Notch1 expression in the hair matrix alone affects the expression of genes both in the hair matrix cells (including *Igfbp2*) and in the dermal papilla (such as *Igfbp3*). It reveals that both cell autonomous and non-autonomous signalings are required for proper proliferation of matrix cells and that a cross talk between two different compartments is involved in this process.[20]

Development of Sebaceous Glands

Invalidation of the Notch signaling pathway in hair follicles alters the differentiation of the sebaceous gland.[10,23] In the skin of mice invalidated for *Notch1* in Msx2 expressing cells (Msx2N1cKO mice), sebaceous glands are detected as swelling in the upper ORS at P1 but they are severely reduced at P12.[23] They contain few mature sebocytes, detected as Oil-Red-O and SCD1 positive cells. In Msx2NIN2cKO mutants, lacking both Notch1 and Notch2 in Msx2 expressing cells, sebaceous glands are missing. Either Notch signaling is required to set aside sebaceous progenitors or defects in sebaceous gland differentiation are a consequence of altered differentiation of the hair follicle.

Invalidation of *RBPJk* in K14 expressing cells severely impairs sebaceous gland differentiation since almost no Oil-Red-O staining is found in the mutant skin, revealing a dependency upon canonical Notch signaling in sebocyte differentiation.[10] However, the sebaceous glands of K5J1cKO mice, in which *Jagged 1* is invalidated in K5 expressing cells, have a normal morphology. As both K14 and K5 are expressed in the basal layer of sebaceous glands,[19,42] it suggests that Jagged1 is not the ligand used by Notch in sebocytes.

When Notch1 is activated in K14 expressing cells ($K14N^{ICD\Delta OP}ER$ mice), the sebaceous glands are enlarged.[19] Whether the enlargement is linked to an increase in cell proliferation or in the sebocyte fate choice remains to be investigated.

Melanocyte Homeostasis

A role for Notch signaling in melanocyte biology was revealed by invalidating either *RBPJk* or *Notch1* and *Notch2* in Tyrosinase expressing cells (TyrRBPJcKO and TyrN1N2cKO mice respectively).[21,30,43] In both cases, the mice had a diluted coat colour at birth, suggesting a role for Notch in the maintenance of melanocyte precursors during embryogenesis. The coat of compound mutants carrying a null allele for *Notch1* or *Notch2* together with the hypormophic *Kit* mutation Kit^{W-v} ($Notch1^{+/-}$; $Kit^{W-v/+}$ or $Notch2^{+/-}$; $Kit^{W-v/+}$) contains larger white spots than $Kit^{W-v/+}$ mice at birth, whereas $Notch1^{+/-}$ and $Notch2^{+/-}$ single mutants have a coat of wild-type colour, revealing an interaction between Notch and KIT signalings in embryonic development of the melanocyte lineage.[44]

Strikingly, during postnatal life, the TyrRBPJcKO and TyrN1N2cKO mutants display an extensive hair graying phenotype associated with a defect in melanocyte renewal in the successive hair cycles. The number of melanocyte stem cells within the

bulge region of mutant hair follicles dwindles with the number of hair cycles, revealing a role for Notch signaling in melanocyte stem cells maintenance. Both Notch1 and 2 are involved in the maintenance as the intensity of the hair greying phenotype depends on the number of *Notch1* and/or *2* alleles deleted.[30] The maintenance defect associates apoptosis and anticipated differentiation of melanocyte stem cells and/or progenitors in the bulge region.[21,43] Transcriptome analysis revealed that *Notch1* and its targets *Hes1* and *Hey1* are overexpressed in melanocyte stem cells compared to melanocyte precursors and differentiated melanocytes.[27] Accordingly, overexpression of *Hes1* in the melanocyte lineage can rescue the hair greying phenotype associated with Notch loss of function, revealing *Hes1* as the major target for Notch signaling in melanocyte stem cells.[21]

Notch is also involved in the control of migration and differentiation of melanocyte progenitors from the bulge to the bulb in anagen hair follicles. Indeed, in TyrRBPJcKO mice, the absence of Notch signaling is associated with the appearance of differentiated melanocytes in ectopic locations in the skin and with an impaired differentiation of melanocyte precursors within the bulb.[43]

Last, in mice invalidated for *Notch1* in Msx2 expressing cells (Msx2N1cKO), expression in the hair bulbs of KITL, the diffusible ligand of KIT, is significantly downregulated.[20] KITL is known to be involved in the migration, proliferation, survival and differentiation of melanocytes and their precursors.[45] Accordingly, the number of melanocytes is reduced in the hair bulbs of Msx2N1cKO mice. As melanocyte survival is dependent upon Notch-mediated activation of *Hes1*,[21] the reduced number could be due to an altered Notch activation in melanocytes of Msx2N1cKO mice. However, Notch activation, as revealed by Hes1 expression, is normal in the melanocytes of Msx2N1cKO mice. Altogether, these results suggest that Notch1 activity in keratinocytes is required for proliferation and/or survival of melanocytes in the hair bulb.[21]

CONCLUSION

The phenotypes associated with loss and gain of function mutations in members of the Notch pathway show how tightly controlled the pathway must be in order to produce a fully developed hair follicle. It raises the question of how the expression of the genes in the Notch pathway is controlled in the hair follicle. The few data available so far deal mostly with the regulation of *Notch1* expression in the hair follicle. On the one hand, Msx2 and Foxn1 proteins are required for expression of *Notch1* in hair matrix cells, their expression being itself dependent on BMP2 and 4.[17] On the other hand, the zinc finger protein GATA-3 is involved in downregulation of *Notch1* expression in the hair matrix.[46] As GATA-3 expression is regulated through BMPs[47] which are also involved in Msx2 and Foxn1-mediated expression of *Notch1*, BMP signaling seems to play a central role in the control of *Notch1* expression levels in the hair matrix. Finally, de novo hair follicles development after β-catenin activation in the epidermis is associated with β-catenin dependent activation of the Notch pathway in hair follicles, suggesting that Notch is placed downstream of β-catenin in hair follicle induction.[19] However, further studies are needed to fully understand the control of Notch1 expression and activation in the matrix and in the bulge and to decipher how the expression of Notch ligands and Notch2 receptor is regulated in the hair follicle.

In postnatal development of the hair follicle, Notch signaling controls cell fate, proliferation, survival and differentiation not only in keratinocytes but also in melanocytes and sebocytes. Notch is involved in complex cross talks between various cell types belonging to

several compartments in the hair follicle.[20] The targets for Notch in hair follicle keratinocytes and the signals involved in the cross talks are poorly known so far. One target gene for Notch signaling in hair follicle differentiation is *Desmoglein 4* (*Dsg4*). Indeed, the phenotype of mice invalidated for *Dsg4* is similar to the phenotype of mice invalidated for *Notch1* and *2* in the hair follicle, in which *Dsg4* expression is reduced.[48] Similarly, the expression of *Igfbp2* and *Kitl* in hair matrix cells and of *Igfbp3* in dermal papilla cells is dependent on Notch signaling.[20] A number of questions remain unanswered so far such as whether *Dsg4*, *Kitl*, *Igfbp2* and *3* are direct targets of Notch, what the other target genes for Notch in hair follicle are and how they exert functions as different as control of hair cycling and control of cell fate, proliferation, survival and differentiation in several cell types.

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CHAPTER 12

NOTCH SIGNALING AND THE DEVELOPING INNER EAR

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Abstract: Sensory hair cells (HCs) and their associated nonsensory supporting cells (SCs) exhibit a typical mosaic pattern in each of the sensory patches in the inner ear. Notch signaling has been considered to conduct the formation of this mosaic pattern through one of its famous functions, known as 'lateral inhibition'. The two Notch ligands Delta-like1 and Jagged2 are believed to act synergistically at the stage of cell diversification in mammals. In addition, many current studies suggest that Notch signaling has another inductive, but not inhibiting, role in the determination of the prosensory region, which precedes the cell diversification of HCs and SCs and Jagged1 is thought to be an essential ligand in this process. Earlier in ear development, the first cell fate determination begins with the delamination of the neuroblasts from the otic epithelium. The delaminated neuroblasts migrate and coalesce to form cochleovestibular ganglion. Notch signaling pathway is thought to function during the delamination through its lateral inhibitory mechanism. Recently, many experiments examining Notch-related gene expression patterns and direct functional analyses of genes have revealed multiple important functions of Notch in inner ear development. Here, we survey a series of studies and discuss the issues that remain to be elucidated in the future.

INTRODUCTION: STRUCTURE AND DEVELOPMENT OF MAMMALIAN INNER EAR

The mammalian inner ear is a finely structured organ, which consists of a turned cochlea, a central vestibule including saccule and utricle, three semicircular canals that are positioned at right angles to each other and a dorsally protruding endolymphatic

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duct and sac. In the inner ear, there are three different kinds of sensory regions: the organ of Corti, lining the cochlear duct; the maculae, contained within the saccule and utricle; the cristae, located at the base of each semicircular canal. The macula sacculi, macula utriculi, and the cristae of semicircular canals are responsible for detecting gravity and linear and angular acceleration, respectively. These five organs are crucial for balance; only one sensory organ, the Organ of Corti, is required for hearing (Fig. 1). All six sensory organs are populated by mechanosensory hair cells (HCs) and their associated supporting cells (SCs). Unfortunately, the production of HCs in the cochlea is completed before birth in mammals. Any subsequent loss of auditory HCs is not corrected, resulting in permanent hearing loss. In the vestibular sensory epithelia of adult mammals, HC regeneration in response to amynoglycoside ototoxicity does occur, although it is extremely limited.[1,2]

The inner ear is derived entirely from the otic placode, which can be initially recognized as a bilateral thickening of the surface ectoderm near the developing hindbrain at embryonic day (E) 8.5 in mice.[3] Once established, the otic placode invaginates to form an enclosed otocyst by E10.5. As the otic vesicle closes, the neuroblasts that will give rise to the cochleovestibular ganglion (CVG) start to delaminate from the

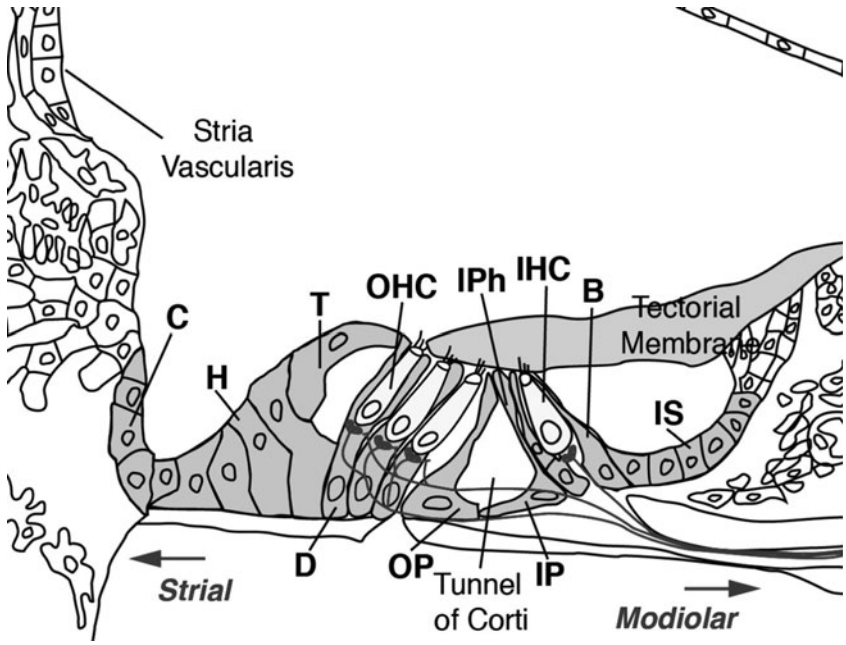


Figure 1. Organ of Corti (the auditory sensory epithelium) in an adult mouse cochlea. A single row of inner hair cells (indicated by IHC) is placed on the modiolar side of the epithelium and three rows of outer hair cells (OHC) are placed on the strial side in the cross-section of the organ of Corti. IHCs are separated from one another by border cells (B) and inner phalangeal cells (IPh), while OHCs are separated by Deiters cells (D). The IHCs and OHCs are separated by the tunnel of Corti, which is formed by inner pillar cells (IP) and outer pillar cells (OP). Thus, the mechanosensory hair cells and their associated supporting cells create a typical mosaic cell pattern. Tectal (T), Hensen (H) and Claudius cells (C) are located at the strial edge and Inner sulcus cells (IS) are located at the modioalar edge of the organ of Corti. The tectorial membrane, which is composed of extracellular matrices, covers IHCs and OHCs in the Organ of Corti, with the longer hairs of the OHCs embedded in it.

ventral region of the otocyst around E9.5 (Fig. 2).[4-6] Almost simultaneously, a narrow extension originates in the dorsomedial region of the otocyst, extending toward the brain to form the endolymphatic duct and sac. Similarly, the cochlear duct forms as an outpocketing from the ventral region of the otocyst and continues to grow until E18. Individual regions in the inner ear under go specialization and develop as the prosensory regions that will contain HCs and SCs (for review see ref. 7). During the growth of the cochlear duct, the cells in the primordial organ of Corti exit the cell cycle between E13.5 and E14.5 to establish a distinct zone of nonproliferating cells delimited by the expression of the cyclin kinase inhibitors $p27^{Kip1}$ and $p19^{Ink4d}$ in the cochlea.[8-11] Subsequently, HCs and SCs differentiate within the sensory primordium to form a precise mosaic cell pattern. Initially, HC progenitors expressing *Math1* appear in the mid-basal region of the cochlea at developmental stages co-incident with or just after terminal mitosis.[12,13] *Math1*, a close homologue of the *Drosophila* proneural gene *atonal*, encodes a member of the bHLH (basic helix-loop-helix) family of transcriptional factors. Math1 is absolutely required for the generation of HC progenitors as well as their survival.[13-15] As the development continues, additional HC progenitors start to appear in a stereotypical wave of differentiation, that is, from the mid-basal region to both apical and basal directions.[12] SC progenitors develop in the area surrounding the HC progenitors, although with a small delay and the robust activation of Notch1 was detected in these cells.[16]

MULTIPLE ROLES OF NOTCH SIGNALING PATHWAY DURING INNER EAR DEVELOPMENT

Hair Cell and Supporting Cell Differentiation

Classic Notch signaling is associated with inhibitory interactions during which the activation of Notch via the ligands expressed on the membranes of a cell that is about to undergo differentiation prevents the adjacent cells from assuming the same cell fate. Thus, this inhibiting interaction between the neighboring cells creates a mosaic cell pattern from initially equivalent cells. The mechanism was originally studied in *Drosophila melanogaster* and *Caenorhabditis elegans* and is usually referred to Notch mediated '*lateral inhibition*'.[17-19] As each HC is surrounded by SCs in the sensory epithelium of the inner ear, it is not surprising that the Notch signaling pathway is thought to play a crucial role in the formation of the HC and SC mosaic through lateral inhibition. In addition, retroviral lineage tracing in the chicken auditory system has identified the existence of two cell clones in which one cell developed as an HC, while the other had developed as an SC, suggesting that the same progenitors give rise to both HCs and SCs.[20] This finding also consistent with a lateral inhibitory mechanism in which different cell types arise from an initially equivalent epithelium.

Expression studies have also demonstrated consistent results; *Notch1* is broadly expressed within the cochlear duct, including the prosensory region.[21] Cells destined to develop as HCs up-regulate expression of two Notch ligands, *Jagged2 (Jag2)* and *Delta-like1 (Dll1)*, with the first signs of ligand expression occurring around E14 in the basal region of the cochlea and subsequently extending towards the apex.[21,22] Within 24 hours of ligand expression, activated Notch1 is observed in adjacent cells[16] as well as expression of at least two Notch target basic helix-loop-helix genes, *Hes1* and *Hes5*

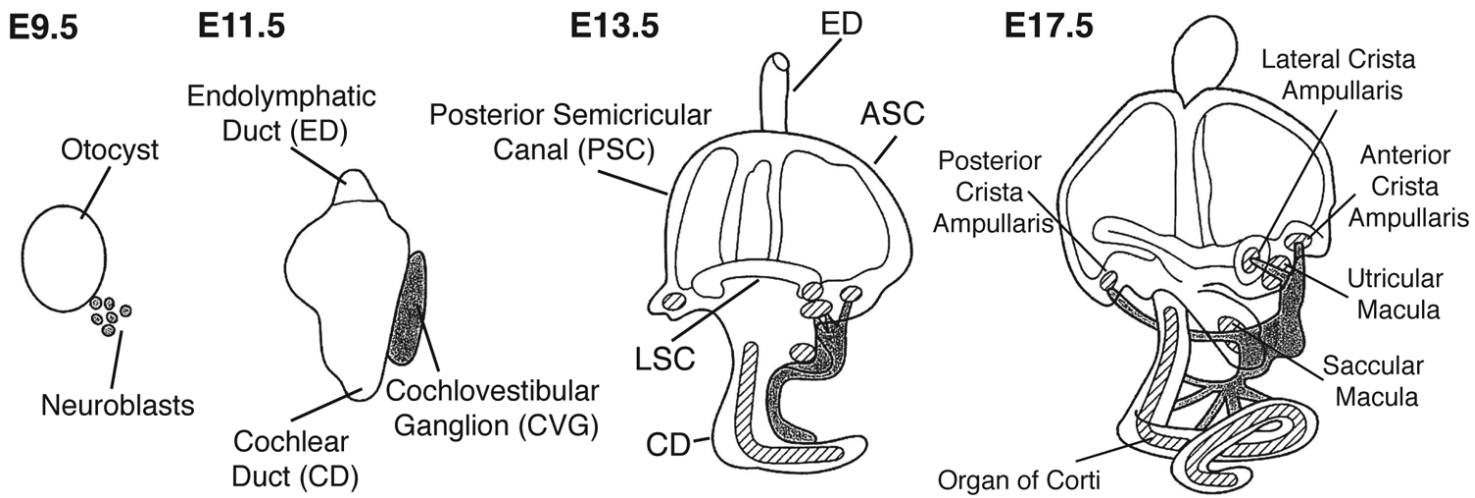


Figure 2. Development of mouse inner ear. Inner ear is derived entirely from the otic placode, which can be initially recognized at embryonic day (E) 8.5 in mice. The otic placode invaginates to form an enclosed otocyst by E10.5. As the otic vesicle closes, neuroblasts that will give rise to the cochleovestibular ganglion (CVG) start to delaminate from the ventral region of otocyst around E9.5. Almost simultaneously, a narrow extension originates in the dorsomedial region of otocyst begins to extend to form an endolymphatic duct and a sac, while the cochlear duct also forms as an outpocketing from the ventral region of the otocyst. At E13.5, neurons in the developing SAG have already started to extend their dendrites that will later contact with HC progenitors in all of the sensory epithelia (the anterior, posterior, lateral crista ampullaris in each semicircular canal; the utricular and saccular macula; the organ of Corti in cochlear duct). The cochlear duct continues to coil and grow until E18 and individual regions in the inner ear under go specialization and develop as the prosensory regions that will contain HCs and SCs. Modified with permission from Kelley MW. Nat Rev Neurosci 2006; 7(11):837-849[7], ©2006 Macmillan Publishers Ltd; and from Morsli H et al. J Neurosci 1998; 18(9):3327-3335[66], ©1998 Society for Neuroscience.

(Hes: Mammalian homologues of Drosophila hairy and Enhancer of split); these cells ultimately develop into SCs.[23,24] Hes1 and Hes5 are supposed to repress the transcription of *Math1*, a crucial gene for HC formation.[25]

A large amount of direct functional evidence exists supporting lateral inhibition in HC and SC differentiation during the development of the mouse inner ear. The deletion of the *Jag2* gene resulted in extra rows of both inner and outer HCs in the cochlea.[21] Similarly, mice lacking one copy of the *Notch1* gene and mice deleted for *Hes1* and *Hes5* deletions exhibited increased number of HCs.[23,26] More recent studies using *Dll1*/*Jag2* double mutant embryos[27] or *Dll1* conditional knockout (cko)mouse embryos[28] have revealed that the Notch ligands *Dll1* and *Jag2* act synergistically to regulate the HC and SC differentiation in a manner consistent with the lateral inhibition theory. Interestingly, the authors suggested that the Notch signaling pathway, via the ligands Dll1 and Jag2, may regulate adequate cellular differentiation through the prevention of excessive cellular proliferation in addition to inducing lateral inhibition.

Determination of Prosensory Region

Jagged1 and Prosensory Determination

Jagged1 (Jag1) and Lunatic Fringe (Lfng) are both components of the Notch signaling pathway, with Jag1 acting as a ligand for Notch and Lfng modulating the activity of some Notch ligands.[29,30] The results of in situ hybridization analyses have shown that *Jag1* mRNA[5,22,31] and *Lfng* mRNA[32] are both expressed in patterns compatible with a role in prosensory formation during inner ear development. Functional analyses also revealed that mice heterozygous for two different *N*-ethyl-*N*-nitrosourea-induced point mutations in *Jag1* gene (*slalom*[33] and *head-turner*)[34] showed mild defects in the sensory organs, especially the reduction of outer HCs in the cochlea. Because embryos homozygous for mutant alleles of the *Jag1* gene do not survive past E11.5, *Jag1* cko mutant mice were created using the Foxg1-Cre mouse line to express Cre-recombinase in early otocysts.[28,35] In *Jag1* cko mice, no HCs or SCs were found at the basal turn of the cochlea, while the number of HCs and SCs was reduced at the apical turn. In the vestibular region, the cristae of semicircular canals were completely lacking and the utricular macula was extremely small. As the saccule and its macula were also mildly affected, the formations of all the six sensory regions of the inner ear were affected to various degrees in *Jag1* cko inner ears. Moreover, markers of the prosensory domain, such as Sox2 and the cyclin-dependent kinase inhibitor $p27^{Kip1}$, are down regulated in *Jag1* cko inner ears. These results suggested that Jag1 is likely to be essential for the determination of the prosensory region in the inner ear.

Role of Notch Signaling in Induction for Prosensory Region

As Jag1 is a Notch ligand, the above-described results imply that Jag1-mediated Notch signaling is essential for establishing the prosensory regions in both of the cochlea and the vestibule. Consistent with this hypothesis, in vitro inhibition of the Notch signaling in cochlear explants with the γ-secretase inhibitor DAPT during early developmental stages reduced the number of HCs and SCs that develop.[36] In gain-of-function studies using the constitutively active, intracellular domain of Notch1 (NICD), NICD induced the expression of prosensory markers when over-expressed in the embryonic mouse

cochlea,[37] and caused the formation of ectopic sensory patches in embryonic chickens.[38] These studies support an inductive role for Notch signaling in the formation of prosensory patches within the inner ear, in addition to its subsequent role in lateral inhibitory signaling that determines the HC versus SC fates later during development. Lewis and coworkers have suggested that a Notch-mediated lateral induction mechanism is involved in the prosensory formation: The activation of Notch1 positively regulates the expression of Jag1 autonomously, gradually strengthening the Notch1 activation and the expression of Jag1 in the prosensory cells.[18,38,39] However, this hypothesis has not been tested in long term with direct gain of function in mammals.

In 2010, a *Cre-/loxP* approach was used to conditionally activate the Notch pathway in nonsensory regions of mouse inner ear epithelia during different stages of otic vesicle morphogenesis.[40] The *Rosa*^{Notch} transgenic mouse yielded a heritable, constitutive co-expression of NICD and nuclear GFP in the presence of Cre-recombinase. The authors selected a *FoxG1Cre* transgenic line that expresses Cre-recombinase in early otic vesicles and several other regions of embryos and found that the broad ectopic activation of Notch at very early stages caused induction of prosensory markers, such as *Sox2* and *Hey1* throughout the entire otic epithelium. Unfortunately, as *Foxg1Cre; Rosa*^{Notch} embryos did not survive past E13.5, they had to use an *hGFAPCCre* transgenic line that provides a more restricted pattern of recombination in the nonsensory region of the developing inner ear during later stages. They observed that at intermediate stages of otic morphogenesis, the activation of Notch1 in a nonsensory region led to the induction of ectopic sensory patches containing HCs and SCs. Moreover, they demonstrated that the activation of Notch1 in isolated nonsensory cells induced the lateral induction of Jag1 in adjacent cells. Another group of the researchers also showed that NICD expression resulted in ectopic HCs and SCs in nonsensory regions of the cochlea and vestibule using a combined Tet-On (tetracycline-on)/Cre induction system in mice.[41] These recent reports substantiate the hypothesis that Jag1-Notch-mediated lateral induction may propagate and maintain the prosensory character during inner ear development in both mice and chicks.

Possible Effectors of the Notch Signaling Pathway in the Context of Early Prosensory Formation

Though Jag1-dependent Notch activation has been revealed to be important for prosensory determination during the earlier stage of cochlear development, little is known about the effectors of the Notch pathway in this context. The Notch effectors *Hey1* and *Hey2* (sometimes referred to as *Hesr1* and *Hesr2*) have been identified as likely mediators of Notch signaling at this stage, mainly based on the expression patterns.[36] However, the presumed functional redundancy and early embryonic lethality made a definitive loss-of-function analysis impossible. Another Notch signaling mediator, *Hes1*, is broadly expressed during the early otocyst stages and may also be involved in defining the prosensory domain.[24,42] A cyclin-dependent kinase inhibitor, p27^{Kip1}, is also known to demarcate the prosensory region in the cochlear primordium, which consists of the sensory progenitors that have completed their terminal mitoses.[9,10] Hes1 reportedly promotes precursor cell proliferation through the transcriptional down-regulation of p27^{Kip1} in the thymus, liver and brain.[43] We showed that *Hes1*, not *Hes5* was weakly expressed at the time of onset of p27^{Kip1} expression and the expression pattern of *Hes1* prior to cell differentiation was similar to that of activated Notch1. In addition, p27^{Kip1} was up-regulated and the number

of BrdU-positive S-phase cells was reduced in the developing cochlear epithelium of *Hes1*-null mice.[24] The results suggest that Hes1, as one of the effectors of the Notch pathway, may contribute to the adequate proliferation of sensory precursor cells via the potential transcriptional down-regulation of $p27^{Kip1}$ expression and may play a pivotal role in the correct prosensory determination.

The HMG-box transcription factor Sox2[44, 45] has also been thought to play an important role in prosensory formation based on its expression pattern during inner ear development and the absent or severely disturbed prosensory patches in two mouse *Sox2* mutant lines, *LCC* and *Ysb*.[46] As the expression of Sox2 is markedly reduced in *Jag1* cko mutant mice,[35] it is supposed that Sox2 may function downstream of the Jag1-Notch1 signaling pathway during prosensory formation. However, the mechanism of the contribution of Sox2 in this context still remains to be elucidated.

Biphasic Regulation of Cell Proliferation by the Notch Signaling Pathway during the Inner Ear Development

We investigated the spatio-temporal expression pattern of activated Notch1 (actN1) during the mouse cochlear development.[24] The results showed that actN1 was diffusely observed in the prosensory region and greater epithelial ridge (GER) during the early developmental stage and Notch1 was activated robustly in SC progenitors at a later stage of cell diversification after E14.5 (Fig. 3). The level of Notch1 signaling switched from a diffuse state to a robust state at the time of cell cycle exit, when the Notch pathway started to promote cell diversification through lateral inhibition.[24] On the other hand, the diffuse pattern of Notch1 activation before E13.5 was closely related to the expression of Hes1 as well as Jag1. Thus, as discussed in the former chapter, the diffuse activation of Notch1 should be critical for the prosensory formation by continuing the adequate proliferation of sensory precursor cells.[24] Interestingly, the higher level of Notch1 activation during the later stage of cochlear development was supposed to inhibit excessive cell division in several studies. A huge increase in HCs was observed in $Dll1^{+/-}$ $Jag2^{-/-}$ cochlea and the authors suspected that their observation resulted from the inhibition of excessive cell division via the Notch pathway during cell diversification.[27] A similar conclusion was drawn in a study examining in vitro culture of mouse fetal cochleae and the reversible inhibition of the Notch-signaling pathway using inhibitors of γ-secretase and TNF-α-converting enzyme.[47] Previous reports have revealed that Notch signaling has similar biphasic roles during the development of the central nervous system (CNS).[48, 49] We propose that the deletion of the *Hes1* allele may suppress cell proliferation during the period of prosensory formation and that excessive cell division might occur during the next cell diversification stage, resulting in the relatively mild increase in HCs observed in $Hes1^{-/-}$ mice at E17.5 in previous studies.[23,24,26]

Delamination of Neural Progenitors

Early during ear development, a subset of cells in the anteroventral region of the otocyst becomes determined as neuroblasts and starts to delaminate from the epithelium at about E9.5 in mice. They migrate and co-alesce to form the primary neurons of the auditory and vestibular systems in cochleovestibular ganglion (CVG).[4-6] Neurogenic basic helix-loop-helix (bHLH) transcriptional factors, such as Neurogenin1 (Ngn1),

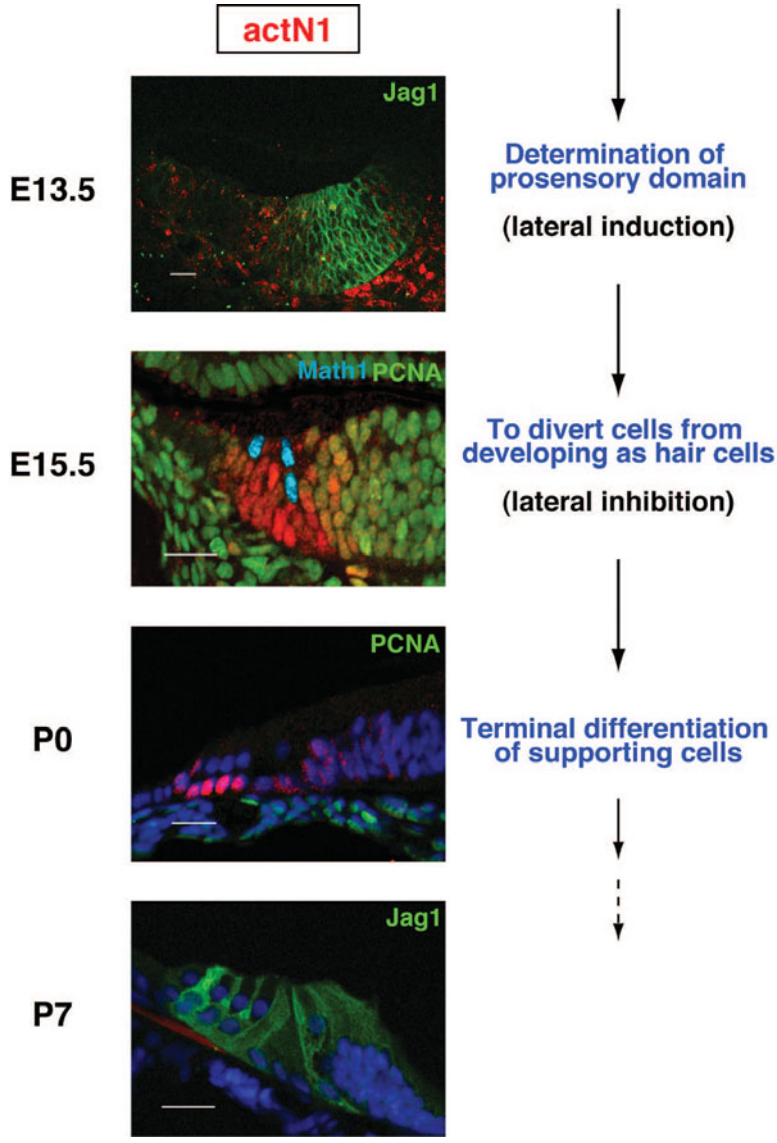


Figure 3. Spatio-temporal activation pattern of Notch1 during the mouse cochlear development. We detected the expression patterns of the activated form of Notch1 (actN1) by immunohistochemistry using an antibody that specifically recognizes the processed form of the intracellular domain of Notch1 (NICD). Notch1 is diffusely activated mainly in the medial region of the cochlear epithelium between E12.5 and E13.5 and the expression pattern of actN1 is closely related to that of Jagged1 (Jag1) (green) at this stage and a positive feedback loop between Notch1 activation and the Jag1 expression is thought to play a role in determining the prosensory cell population through Notch-mediated lateral induction. Notch1 is robustly activated in cells surrounding Math1-positive (light blue) hair cell progenitors during later stage, for example, at E15.5 in the figure. Notch1 activation at this stage is thought to divert cells from developing into hair cells (HCs) through lateral inhibition. Notch1 is strongly but transiently activated at the perinatal stage in the supporting cell (SC) lineage, so Notch1 may play an instructive role in the terminal differentiation and maturation of SCs. Modified with permission from Murata J et al. J Comp Neurol 2006; 497(3):502-518,[16] ©2006 John Wiley and Sons.

NeuroD1 (NeuD1) and Math1, are crucial for in the development of various nervous systems. In the inner ear, Ngn1[50, 51] and NeuD1[52] are required for the generation or delamination and survival of CVG neurons respectively. Lateral inhibition has been suppered to be involved in this process as well as in diversification of HCs and SCs at the later stage during the inner ear development. That is, Dll1-expressing cells delaminate as neuroblasts from the early otocyst and Notch1 is activated in other cells surrounding the Dll1-positive cells. The differentiation of these surrounding cells into neuroblasts are inhibited and they instead become sensory or nonsensory epithelial cells. The volume of the CVG reportedly increased, with the saccular and utricular maculae being lost or severely reduced in *Dll1* cko mice.[28] The phenomenon can be interpreted as indication that when the lateral inhibition failed via the deletion of *Dll1,* an excessive proportion of cells was diversified towards a neuronal fate, resulting in the depletion of the prosensory population in the anteroventral otic epithelium, which is supposed to develop as maculae.

In contrast, a recent study demonstrated that the CVG was smaller in *Jag1* cko mice, with significantly fewer neuroblasts (Ngn1-positive cells) within the otic vesicle.[54] The authors supposed that Jag1 might be important for the specification and maintenance of 'neurosensory progenitors' given the decrease in both sensory and neural progenitors in *Jag1* cko inner ears. Lineage experiments indicate that sensory cells in the maculae and neuronal cells can share a common progenitor in chik.[56] In addition, fate-mapping experiments have demonstrated that not all Ngn1-positive cells become neuroblasts, with some becoming sensory and nonsensory cells in the macular regions of mice.[54] These data support the existence of a neurosensory progenitor and may reinforce the hypothesis that Notch-dependent lateral inhibition among the neurosensory progenitors plays a crucial role in neuroblast delamination.

NOTCH SIGNALING PATHWAY AND THE INNER EAR REGENERATION

The production of HCs in the cochlea is finished before birth in mammals. Any subsequent loss of auditory HCs is not compensated, resulting in irreversible hearing loss. In contrast, many nonmammalian vertebrates readily regenerate HCs into adulthood and HC regeneration has been most thoroughly studied in birds (see review ref. 57). In birds, when acoustic trauma or ototoxic drugs destroy HCs, SCs give rise to new HCs in two different ways. First, SCs are directly converted into new HCs through a method described as *transdifferentation*.[57, 58] A few days later, SCs adjacent to the dying HCs re-enter the cell cycle, dividing asymmetrically to generate new HCs and SCs.[60, 61] In the normally quiescent bird auditory epithelium, HC regeneration seems to re-activate developmental programs and the Notch signaling pathway is probably one of the best candidates for investigation. During development, Notch receptor activation suppresses HC diversification through the up-regulation of the *Hes* genes, which are potent inhibitors of *Math1*. As a result, the control of the Notch pathway, such as through the deletion of Notch ligands, leads to supernumerary HCs. Correspondingly, during zebrafish lateral line[62] and chick basilar papilla (BP; auditory epithelium) HC regeneration,[63] the inhibition of the Notch signaling pathway increases the production of new HCs. Daudet and co-researchers showed that the blockade of Notch signaling by DAPT had no direct effect on SC division after HC damage in organ cultures of chick BP.[63] However, DAPT caused the excessive regeneration of HCs at the expense of SCs, through both mitotic and nonmitotic mechanisms with the up-regulation of *Delta1*

and *Hes5* genes. They also confirmed that the inhibition of γ-secretase in undamaged BP does not trigger HC production. Conversely, the over expression of NICD in SCs after damage caused them to maintain their phenotype and inhibited HC regeneration. These data imply that Notch signaling controls the switches of the cell diversification, but may not directly regulate the cell division in the regeneration of BP. The authors supposed that another signal (Q signal; signal for quiescence), which is independent of Notch signaling (N signal), functions during quiescence and regeneration and inhibits SCs from direct transdifferentiation into HCs and from cell division to product HC and SC progenitors.

In mammals, the applications of DAPT to explants of embryonic and neonatal organ of Corti resulted in the robust production of supernumerary HCs in the case of mice.[47,64] Continuous inhibition through the application of DAPT also seems to induce the mitogenic proliferation of SCs in the cultures of embyronic organ of corti.[47] However, experiments on damaged adult guinea pig organs of Corti are disappointing; local application of a γ-secretase inhibitor, MDL28170, resulted in the formation of very limited ectopic HCs in the inner sulcus region.[65] The results might imply that when HCs are injured in adult mammals, the Q-signal that has been described in the case of chicks cannot be adequately weakened by some unknown factors. We might be able to elucidate these factors by investigating other signaling pathways that are act reciprocally with the Notch signaling pathway during inner ear development.

CONCLUSION

Auditory and vestibular sensory organs in the inner ear are composed of mechanosensory hair cells (HCs) and associated supporting cells (SCs). HCs are not neighboring each other and are separated by SCs. As a result, they form a typical mosaic cell pattern, which have intrigued the researchers to think that Notch mediated inhibitory mechanism called 'lateral inhibition' should play a pivotal role in HC and SC differentiation during inner ear development. Recent about 15 years' progress of molecular and developmental biology have revealed that the Notch signaling pathway have multiple important roles in developing inner ear, including the determination of prosensory region, the regulation of cell proliferation as well as the cell diversifications. Unfortunately, mammalian auditory epithelium do not have regenerative capacity when they have been injured by noise or modern drugs, contrasting to that of non-mammalian vertebrates including birds, in which HC regeneration seems to reactivate the developmental programs. Some researchers tried to induce HC regeneration in damaged adult mammalian cochlea via manipulating the Notch signaling pathway, for example, by applying γ-secretase inhibitors. However, they have not succeeded in the HC restoration yet. By investigating other factors and signaling pathways that act reciprocally with the Notch signaling pathway during inner ear development, we might be able to make progress in the regenerative medicine for hearing deficient.

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CHAPTER 13

NOTCH SIGNALING IN CANCER STEM CELLS

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Abstract: Subpopulations of cancer cells with stem cell-like characteristics, termed cancer stem cells, have been identified in a wide range of human cancers. Cancer stem cells are defined by their ability to self-renew as well as recapitulate the original heterogeneity of cancer cells in culture and in serial xenotransplants. Not only are cancer stem cells highly tumorigenic, but these cells are implicated in tumor resistance to conventional chemotherapy and radiotherapy, thus highlighting their significance as therapeutic targets. Considerable similarities have been found between cancer stem cells and normal stem cells on their dependence on certain signaling pathways. More specifically, the core stem cell signaling pathways, such as the Wnt, Notch and Hedgehog pathways, also critically regulate the self-renewal and survival of cancer stem cells. While the oncogenic functions of Notch pathway have been well documented, its role in cancer stem cells is just emerging. In this chapter, we will discuss recent advances in cancer stem cell research and highlight the therapeutic potential of targeting Notch in cancer stem cells.

INTRODUCTION

Treatment of tumors by current anti-cancer therapeutics, which primarily focus on tumor shrinkage, is mostly palliative. Recent advances in cancer stem cell research suggest that we should revisit the principles of anti-cancer drugs in light of the intrinsic tumor cell heterogeneity. Albeit the percentage of cancer stem cells is often very low, this subpopulation may exclusively retain the tumorigenic potential of the entire tumor.[1] The cancer stem cell model further predicts that these cells are required to give rise to

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macrometastasis and recurrent tumors that are primary causes of cancer-related death. Additionally, cancer stem cells are found to be resistant to a variety of conventional chemoradiotherapies.[2-4] These unique biological features of cancer stem cells suggest that it is imperative to develop innovative approaches that can effectively eradicate these cells.

The Notch signaling pathway plays pleiotropic roles in embryonic development and in homeostasis of adult tissues through regulation of cell-fate determination of stem cell and progenitors (reviewed in other chapters). Aberrant Notch activation has been documented in a wide range of human cancers, such as breast cancer[5] and T-cell acute lymphoblastic leukemia (T-ALL).[6] The concept of targeting the Notch signaling pathway as a therapeutic strategy for treating cancer has attracted increasing interest.[7,8] More importantly, several recent studies suggest that Notch signaling is important for cell survival and self-renewal mechanisms of cancer stem cells.[9,10] In this chapter, we will first discuss recent advances in our understanding of the cancer stem cell model and its clinical implications. In the second part of this chapter, we will focus on the roles that Notch play in self-renewal, survival and therapeutic resistance of cancer stem cells.

THE CANCER STEM CELL MODEL

Tumors are heterogeneous morphologically as well as functionally. Two competing models have been developed to explain this heterogeneity. The stochastic model argues that tumor heterogeneity arises upon accumulation of random genetic and epigenetic changes and is influenced by microenvironmental factors.[11] According to this model, the tumor-initiating cells are randomly selected and thus cannot be enriched from the bulk population. In contrast, the cancer stem cell model suggests that tumors are organized in a hierarchical manner that a small subpopulation of cancer cells with stem cell-like characteristics drives tumor progression and gives rise to multiple lineages of differentiated progenies.[11] This model predicts that cancer stem cells can be prospectively enriched from the bulk tumor population as these cells are biologically distinct from the differentiated cancer cells. It needs to be emphasized that the cancer stem cell model does not exclude the impact of genomic instability, thus cancer stem cells should be referred as a dynamic concept.

Cancer stem cells are defined by their ability to continuously self-renew and to generate differentiated progenies. The concept of stem-like cells in cancer has been postulated decades ago. However, the existence of cancer stem cells was not experimentally confirmed until 1994, when John Dick's group enriched the leukemia-initiating cells from acute myeloid leukemia (AML) specimens through selection for cell surface markers $CD34^{+}/CD38^{-}$.[12] Of note, this cell surface phenotype of AML stem cells mimics that of normal hematopoietic progenitors. Based on the same strategy, cancer stem cells of solid tumors were first identified in breast cancer by Michael Clarke and colleagues in 2003.[13] The tumorigenic potential was highly enriched in a small percentage of breast cancer cells with the phenotype of $CD44^{+}/CD24^{-}$/low/Lineage-. As few as 100 breast cancer stem cells could generate xenograft tumors in immunocompromised mice, whereas half million remaining cancer cells failed to do so.[13] Additionally, this subpopulation could be serially passed as xenografts transplanted into the mammary fat pad of immunocompromised mice and consistently recapitulate the original heterogeneity of the tumor.[13] Thus these cells met the criteria for cancer stem cells. Shortly after, Peter Dirk and colleagues described the cancer stem cells in gliomas by selection for CD133 (Prominin-1), a cell

surface marker originally identified in hematopoietic progenitors.[14,15] These pioneering studies were followed by a number of reports that identified cancer stem cells from a wide range of tumors, including colon,[16,17] pancreatic,[18] prostate,[19] lung,[20] liver[21] and head and neck cancer.[22]

In addition to selection for cell surface molecular signatures, cancer stem cells can be enriched based on their functional features, such as the ability to efflux DNA-binding dyes and the enzymatic activity of aldehyde dehydrogenase (ALDH). This first approach highlights a small subgroup of the so-called side population (SP) cells that effectively efflux fluorescent DNA-binding dyes, e.g., Hoechst 33342.[23] SP cells with cancer stem cell-like characteristics have been derived from a variety of human tumors.[23] These cells often express high levels of the ATP-binding cassette (ABC) transporter family membrane proteins, such as MDR1 and ABCG2, which not only promote efflux of the dyes but also endow SP cells with resistance to chemotherapy.[23] One of these ABC proteins, ABCB5 has been identified as a marker for melanoma cancer stem cells.[24] Additionally, tumor-initiating activities can be enriched based on the ALDEFLUOR assay that detects high ALDH activity, which is a feature originally found in normal hematopoietic progenitor cells.[25,26] For example, ALDEFLUOR-positive populations enriched from both normal mammary epithelia and breast cancer biopsy samples display stem cell/progenitor properties.[27] Interestingly, a small subset of breast cancer cells that are double positive for the ALDEFLUOR assay and for the $CD44^+/CD24^-$/low/Lineage- phenotype demonstrated higher tumorigenic potential than either singly positive population and could generate tumor xenografts from as few as 20 cells.[27] The levels of ALDH1, the most active ALDH enzyme, correlate with clinical outcome of breast cancer patients, highlighting the clinical significance of ALDH and the breast cancer stem cell population.[27] When studies of normal and malignant stem cells have been limited by the availability of specific cell surface markers, the dye exclusion assay and the ALDEFLUOR assay have provided important alternatives.

CLINICAL IMPLICATIONS OF CANCER STEM CELLS

The concept that many cancers are sustained by a subpopulation of cancer stem cells has stirred tremendous interests in the cancer research community. This model has profound implications with regard to the development of new diagnostic and therapeutic approaches for treating cancer. A key question remained to be answered is whether the frequency and other biological features of cancer stem cells can predict the clinical outcome of the corresponding tumors. Although some controversies exist, several recent studies have provided compelling results. As aforementioned, ALDH activity not only identified the cancer stem cell fraction in breast cancer, but also predicted poor patient outcome.[27] Michael Clarke and colleagues have shown that the gene expression profiles of breast cancer stem cells predicted overall and metastasis-free survival.[28] Another study evaluated 136 primary or recurrent paraffin-embedded breast cancer specimens and provided data that the frequency of the $CD44^+/CD24^-$/low phenotype did not predict clinical outcome but might be associated with distant metastasis.[29] Study of acute myeloid leukemia (AML) also suggests that the frequency of the $CD34^+/CD38^-$ AML stem cells correlates with the ability of AML to engraft immunocompromised mice as well as poor clinical outcome.[30] Similarly, the proportion of $CD133^+$ cancer stem cells in gliomas has been found to be a prognostic predictor for duration of progression-free survival and overall

survival independent of tumor grade, extent of resection, or patient age.[31] The frequency of $CD133^+$ cells also correlates with tumor recurrence and malignant progression in WHO Grade II and III gliomas.[31] Although these population-based studies cannot fully address the cellular identity as the definition of cancer stem cells remains functional, these results have highlighted the clinical relevance of cancer stem cells. Additionally, a rapidly growing body of evidence has demonstrated that cancer stem cells from a variety sources are refractory to many conventional chemotherapies and radiotherapies.[2,32] Given the strong tumorigenic potential of cancer stem cells, it is reasonable to speculate that the initial tumor response to conventional cancer therapy often reflects death of differentiated cancer cells, but insufficient killing of cancer stem cells eventually leads to tumor recurrence. Therefore, the cancer stem cell model predicts that these cells must be effectively eradicated to achieve sustained tumor control.

CANCER STEM CELLS AND CHEMORESISTANCE

Clinical failure of cancer treatment is often associated with therapeutic resistance. Typically, the recurrent tumors are more resistant to the original treatments.[32] In the conventional view of therapeutic resistance, some tumor cells randomly acquire genetic mutations that endow resistance to the treatment(s) and subsequently repopulate the tumor(s). In contrast, the cancer stem cell model suggests that recurrent tumors originate from cancer stem cells that are intrinsically resistant to the treatment. Increasing evidence exists that cancer stem cells are resistant to a variety of conventional chemotherapies and radiotherapies and the percentage of cancer stem cells increases following treatments.[3,33] A recent study comparing paired breast cancer biopsy specimens before or after treatment reported that the proportion of the $CD44^+/CD24^{-/low}$ cancer stem cells is significantly increased in the specimens treated with neoadjuvant chemotherapy.[34] In contrast, this subpopulation was not altered in tumor treated with lapatinib (an inhibitor of tyrosine kinases including the epidermal growth factor receptor and HER2) alone or in combination with chemotherapy, suggesting that pathway-specific inhibitors may prove to be more effective than conventional chemotherapy to target cancer stem cells.[34] In both treatment groups, the percentage of $CD44^+/CD24^{-/low}$ cells correlated with mammosphere-forming capacity in culture and tumorigenic potential in xenotransplantation assays, thus validating these cells as cancer stem cells.[34] Similarly, the $CD133^-$-positive cancer stem cells in glioblastoma also resist several common chemotherapeutic agents.[35] Pancreatic cancer stem cells with the $CD133^+$ phenotype demonstrated reduced apoptosis in response to the standard chemotherapy drug gemcitabine in comparison to the matched nonstem cancer and rapidly resumed proliferation following drug withdrawn.[36] Additionally, $CD133^+$ colon cancer stem cells display resistance to fluorouracil or oxaliplatin, which can be attenuated by neutralizing antibody against the inteleukin-4 receptor.[37] Recently, a retrospective study addressed the chemoresistance of cancer stem cells by analyzing the stem cell-like phenotypes of drug-resistant lung cancer cells.[38] In this study, the cells survived treatments of different chemotherapeutic reagents, including doxorubicin, cisplatin or etoposide, displayed upregulation of stem cell markers and enhanced tumorigenic and metastatic potential in xenograft assays. The authors also suggested that chemotherapies might have stimulated the expansion of cancer stem cells, potentially through changes of tumor microenvironment.

Most chemotherapeutic reagents are designed to target rapidly-dividing cells. Normally most somatic stem cells are quiescent and thus are protected from these drugs.

Hematopoietic cancers comprise quiescent cancer stem cells and rapid-dividing progenitor cells that resemble the hierarchy found in the normal hematopoietic system.[39,40] Slow proliferation may make the hematopoietic cancer stem cells relatively insensitive to chemotherapeutic reagents. However, cancer stem cells derived from solid tumors often demonstrate strong proliferative capacity in culture, possibly due to the high concentration of growth factors presented in media. It remains an open question how the proliferation index of solid tumor stem cells is implicated in therapeutic resistance in vivo. Our recent study in addition to results from other groups suggests that glioma stem cells express high levels of c-Myc in vitro as well as in vivo, implying that these cells were actively proliferating.[41,42] Another potential drug-resistant mechanism shared by both normal and malignant stem cells is their ability to efflux toxic reagents, which is exemplified by the "side-population" cells.[23] Multiple mechanisms may combinatorially endow cancer stem cells high resistance to chemotherapeutic reagents. Glioblastomas cancer stem cells enriched for the CD133 marker overexpress the ABC transporter protein ABCG2 and the DNA repair protein MGMT.[43] Additionally, high levels of pro-survival genes, such as Bcl-2, Bcl-xL, FLIP and IAP family proteins, may further strength the chemoresistance of these cancer stem cells.[43]

CANCER STEM CELLS AND RADIORESISTANCE

Several recent studies also suggest that cancer stem cells are more resistant to radiation than the matched nonstem cancer cells. In malignant glioma, radiation exposure increases the fraction of the $CD133^+$ cancer stem cells in culture as well as in xenografts, consistent with enhanced radioresistance of these cells.[44] The higher radiation resistance found in the glioma stem cells appears to correlate with preferential activation of check point kinases and more efficient DNA damage repair.[44] $CD133^+$ cells isolated from the Daoy medulloblastoma cell line also demonstrated stronger radioresistance than the differentiated glioma cells.[45] Additional evidence comes from studies of breast cancer. Breast cancer cells (MCF-7 and MDA-MB-231) propagated as mammospheres were found to be enriched for the $CD44^+/CD24^-$/low stem cell-like population and demonstrated higher clonogenic survival following exposure to radiation than the parental cell lines grown as monolayer cultures.[46] Of note, expression of Jagged-1 and levels of the intracellular domain of Notch moderately increased in response to radiation exposure, suggesting a potential role of Notch signaling in radiation response of breast cancer stem cells.[46] Both Michael Clarke's and Frank Pajonk's groups provided data that reactive oxygen species (ROS) levels were lower in the breast cancer stem cells than the corresponding nonstem cancer cells, which represents a mechanism shared with the normal breast stem cells.[46,47] Reactive oxygen species are important mediators for ionizing radiation-induced DNA damage and subsequent cell death,[48] thus explained why less radiation-induced DNA damage was found in breast cancer stem cells. Additionally, the Wnt/β-catenin pathways may be involved in radioresistance of breast cancer stem cells.[49] Woodward and colleagues showed that radiation increased the percentage of side-population cells with stem cell-like phenotypes in primary murine mammary epithelial culture as well as in the MCF-7 breast cancer cell line. In these side population cells, Wnt was preferentially activated in response to radiation compared with the differentiated cancer cells and upregulated expression of the anti-apoptotic gene, survivin.[49]

NOTCH SIGNALING IN CANCER STEM CELLS

As mentioned above, significant similarities have been found between normal stem cells and cancer stem cells with regard to the signaling pathways that control their self-renewal, survival and cell-fate determination. On the top of this list are the developmental pathways controlled by Wnt, Notch and Hedgehog.[50] Recent discoveries that these pathways may play instrumental roles in cancer stem cells have stimulated substantial interest for their potential as therapeutic targets and diagnostic predictors.

The oncogenic role of Notch is highlighted by the presence of activating mutations and amplifications of Notch pathway elements in a variety of human tumors, including lymphoma, breast cancer, lung cancer, head and neck cancer, pancreatic cancer, colon carcinoma, osteosarcoma and glioblastoma.[51,52] In particular, activating mutations of Notch1 are reported in approximately 50% of human T-cell acute lymphoblastic leukemia (T-ALL), making it the most frequent oncogenic event in this disease.[8] The Notch inhibitor Numb is lost in approximately 50% of human mammary carcinomas.[53,54] Upregulated expression of Notch pathway components is also found in glioblastoma multiforme cell lines and patient samples.[55,56] In a context-dependent manner, Notch signaling may also prevent tumorigenesis. For example, loss of Notch1 or expression of a dominant negative MAML1 mutant in mouse epidermis induces hyperplasia followed by development of skin tumors.[57,58] In contrast to the prevalent de-differentiation role of Notch in stem cells, Notch activity promotes terminal differentiation in keratinocytes through upregulation of p21WAF1 and caspase-3.[59,60] Taken together, these findings suggest that the tumorigenic or tumor suppressive activities of Notch in different tumor types reflect its roles to promote or repress the undifferentiated status of stem/progenitor cells in the corresponding tissues.

The study of the Notch signaling pathway in cancer stem cells is still in its infancy. This pathway has been most extensively studied in the developing and adult nervous system for its pleiotropic functions to maintain the pools of neural stem/progenitor cells and to direct lineage specification.[61] Many studies have shown that Notch activation promotes self-renewal and survival of neural stem/progenitor cells and blocks differentiation. Conversely, withdrawn of Notch signals leads to diminished pools of progenitor cells and increased neuronal differentiation.[10,62] Several Notch components have been found to be overexpressed in glioma cell lines and surgical specimens, such as Notch1, Notch3 and Notch ligands, Delta-like-1 and Jagged-1.[55,56] Targeting any of these Notch pathway components impaired proliferation or survival of glioma cell lines,[56] suggesting that an integrated Notch signaling pathway is important in gliomas. Focusing on the cancer stem cell fraction, Fan and Eberhart reported that Notch inhibition by a γ-secretase inhibitor (GSI-18) depleted the $CD133^+$ stem-like subpopulation derived from medulloblastoma cell lines and abolished xenograft formation.[63] GSI-18 induced 10 fold stronger apoptosis in cells expressing the stem cell marker Nestin in comparison to the Nestin-negative cells, suggesting a preferential Notch dependence of the cancer stem cell subpopulation.[63] The same group recently extended their study to the $CD133^+$ cancer stem cells derived from glioblastoma specimens and reported similar findings.[64]

The role of Notch signaling has also been examined in normal and malignant stem/progenitor cells derived from mammary tissue.[65] Notch activation stimulated expansion of human mammary stem cells in culture as shown by significantly increased mammosphere formation and a myoepithelial lineage-specific commitment.[66] In contrast, fully differentiated mammary epithelial cells did not respond to modulation of Notch signaling.[66] However, genetic mouse models suggest that Notch activation favored expansion of luminal progenitors and prevent inappropriate expansion of mammary stem cells and myoepithelial

progenitors.[67,68] In breast cancer, overexpression of Notch1 or the Notch ligand Jagged-1 has been found to predict poor overall survival,[69,70] highlighting the critical role of Notch in this disease. Overexpression of epidermal growth factor receptor Type 2 (HER2) is frequent in breast cancer and is associated with poor prognosis. [71] Notch activates HER2 expression in spheroid cells with stem cell-like phenotypes derived from breast cancer cell lines and HER2 activity is essential for survival and self-renewal of these cells.[71] Additionally, high levels of the intracellular domain of Notch1 correlate with shorter disease-free survival in patients with ductal carcinoma in situ (DCIS).[9] Notch inhibition by a γ-secretase inhibitor DAPT or a Notch4 neutralizing antibody reduces the mammosphere-forming capacity of cells derived from DCIS.[9] Pajonk and colleagues also provide evidence that recombinant human erythropoietin promotes self-renewal and survival of breast cancer stem cells in a Notch-dependent manner.[72] Additionally, interleukin-6 signaling pathway may rely upon Notch-3 activity to maintain self-renewal and promote a hypoxia-resistant phenotype in normal mammary progenitors as well as mammary cancer stem cells.[73,74] In addition to breast and brain tumors, Notch signaling is also found to be implicated in self-renewal and survival of the $CD34^{+}/CD38^{-}$ cancer stem cells of acute myeloid leukemia, as shown by decreased colony-forming activity in the presence of DAPT.[75]

Although the data focusing on the role of Notch in cancer stem cells are limited, most studies agree that Notch activation favors self-renewal and survival of these cells. This observation is in line with the central function of Notch in normal stem/progenitor cells. Activation of Notch by upstream oncogenic stimulus and microenvironmental cues is anticipated to stimulate expansion of cancer stem cell pools and thus facilitate tumor progression. Taken together, current results suggest a role of Notch at the center of regulatory network of the "stemness" of cancer stem cells, thus targeting Notch is likely to provide sustained benefits for cancer treatment. Our recent findings that Notch regulates radiation resistance of glioma stem cells further underscores this therapeutic paradigm and suggests that synergistic benefits might be generated by combining Notch inhibition with conventional therapeutic approaches.

NOTCH PROTECTS CANCER STEM CELLS FROM RADIATION

Malignant glioma is among the most lethal human malignancies and is routinely treated by radiation.[76-78] However, malignant glioma is extremely resistant to radiotherapy and recurrence is almost inevitable, suggesting inadequate killing of tumorigenic cells. As aforementioned, the cancer stem cell fraction of malignant glioma displays stronger radioresistance in vitro than the matched nonstem glioma cells, suggesting that recurrent gliomas may be driven by the cancer stem cells that survive radiotherapy.[44] Targeting the molecular mechanisms underlying the radioresistance of glioma stem cells thus holds promise to improve care for those with this lethal disease. Previous studies have shown that radiation induced moderate activation of Notch signaling in the $CD44^{+}/CD24^{-/low}$ breast cancer stem cells[46] and endothelial cells,[79] suggesting that Notch is potentially involved in the radiation response. We recently interrogated the role of Notch signaling in the radiation response of the $CD133^{+}$ cancer stem cells derived from malignant glioma specimens and determined that Notch is critical for radioresistance in these cells. [80] Notch inhibition by γ-secretase inhibitors or by RNA-based silencing of either Notch1 of Notch2 markedly increased the radiosensitivity of glioma stem cells but not that of differentiated glioma cells. Conversely, Notch activation by expression of the constitutively active

intracellular domains of Notch1 or Notch2 protected cells against radiation even in the presence of γ-secretase inhibitors. The most significant biological consequence of Notch inhibition in irradiated glioma stem cells was markedly increased apoptotic cell death as shown by stronger activation of caspase 3/7 and positive labeling of the cell surface apoptotic marker, Annexin V. In line with these observations, the radioprotective functions of Notch required an intact PI3K/Akt pathway. Interestingly, many studies interrogating the pro-survival function of Notch signaling have identified the PI3K/Akt pathway as the key downstream mediator of Notch,[62,81-86] although the mechanisms through which Notch activates Akt may vary in different cell types and can be independent of transcription. Our laboratory recently demonstrated that glioma stem cells were highly sensitive to Akt inhibition, but the impact of such inhibition on differentiated glioma cells was less significant.[87] Therefore, the radioprotective role of Notch in glioma stem cells may reflect these cells' preferential dependence on Akt activity,[87] particularly in response to radiation exposure. A recent study categorized advance gliomas based on functional genomics and linked expression of neural stem cell markers to short patient survival.[88] Notably, this study also shows that activation of Notch and Akt signaling are two strong predictors for poor prognosis.[88] In line with these reports, our recent findings further highlight the potential utility of targeting the Notch-PI3K/Akt pathway for glioma treatment.[80]

CONCLUSION

Although the validity of the cancer stem cell model, the frequency of cancer stem cells and their clinical relevance still remain open questions, cancer stem cell research over the past decade has substantially changed our perspective on the diagnosis and treatment of cancer. It might be too early to predict that cancer stem cell-focused research will eventually lead to improved treatment of cancer. Nevertheless, recent studies have highlighted the anti-cancer potentials of the developmental pathways known to be important for normal stem cells, such as Notch, Wnt, Hedgehog, Bmi, etc., based on their similar roles in cancer stem cells.[89,90] Among these pathways, Notch is of particular interest due to its pluripotent functions in various cancer stem cells and the availability of many small molecule inhibitors (γ-secretase inhibitors) that have been developed and examined in preclinical and clinical studies for other indications.[91]

The studies discussed in this chapter have collectively demonstrated that inhibiting Notch may directly target the "stemness" of cancer stem cells, as shown by decreased self-renewal, survival and consequently loss of tumorigenic potential. As discussed in other chapters, small molecule inhibitors of the γ-secretase complex have been widely used as research tools and therapeutic reagents targeting Notch pathway based on the essential role of γ-secretase complex to mediate Notch activation.[92,93] Most γ-secretase inhibitors are originally developed for the potential treatment of Alzheimer's disease, because the γ-secretase complex also mediates production of β-amyloid peptides, the precursor of amyloid plaques found in Alzheimer's disease.[94] These molecules have also been actively examined for their anti-cancer efficacy in a variety of tumor types, such as T-ALL,[95] Kaposi's sarcoma,[96] medulloblastoma,[97] intestinal adenoma.[7] Currently, the clinical trial of MK-0752, a γ-secretase inhibitor made by Merck, is underway for breast cancer treatment in combination with chemotherapeutic reagents, such as docetaxel, tamoxifen or letrozole (clinicaltrials.gov). Additionally, systemic administration of γ-secretase inhibitors has been shown to reduce the activity of γ-secretase complex in

the central nervous system of experimental animals and patients, thus validating their ability of penetrating the blood-brain barrier.[98-102] The potential of γ-secretase inhibitors in treating brain tumors are further highlighted by our recent discovery that these molecules compromise radiation resistance in cancer stem cells derived from malignant gliomas.[80]

Future efforts will focus on preclinical studies and translation of Notch targeting agents into clinic approaches in a context of conventional radiotherapy or chemotherapy. To this end, it is important to improve our understanding of mechanisms through which the Notch signaling pathway controls self-renewal and survival in different cancer stem cell types. It will also require comprehensive knowledge of how Notch cross-talks with other signaling pathways, such as the PI3K/Akt pathway,[84,85] the DNA damage response pathway,[80] the NF-κB pathway,[103] for designing combinatorial treatments that effective kill cancer stem cells. Targeting cancer stem cell-specific pathways, such as Notch, may have limited impact on the differentiated cancer cells,[80] thus may not induce immediate tumor shrinkage like conventional therapies do. When evaluate the therapeutic benefits of cancer stem cell-specific approaches, it is important to assess the long-term survival benefit in addition to immediate change of tumor volume.

Finally, individual Notch receptors may play different and sometime even opposite roles in the same tumor type.[104] For example, Notch1 has been shown to have tumor suppressor-like activities in multiple tumor types, such as keratinocyte cancer, mesothelioma and medulloblastoma, where Notch2 can be oncogenic.[58,104-106] Because of this, more selective Notch inhibitory agents are being developed, such as monoclonal antibodies against Notch ligands or receptors.[107,108] The ultimate goals will be to develop selective Notch inhibitory strategies that effectively eradicate cancer stem cells or maximally sensitize these cells to conventional chemo/radiotherapy, while minimizing adverse effects on normal stem/progenitor cells.

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CHAPTER 14

NOTCH SIGNALING PATHWAY AND CANCER METASTASIS

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Abstract Cancer metastasis is the leading cause of cancer-related deaths all over the world at present. Accumulated researches have demonstrated that cancer metastasis is composed of a series of successive incidents, mainly including epithelial-mesenchymal transition (EMT), malignant cell migration, resistance to anoikis, and angiogenesis and lymphangiogenesis processes. However, the complicated cellular and molecular mechanisms underlying and modulating these processes have not been well elucidated. Thus, studies on cancer metastasis mechanism may propose possibilities to therapeutically interfere with signaling pathways required for each step of cancer metastasis, therefore inhibiting the outgrowth of distant metastasis of tumors. Recent insights have linked the Notch signaling pathway, a critical pathways governing embryonic development and maintaining tumor stemness, to cancer metastasis. This chapter highlights the current evidence for aberration of the Notch signaling in metastasis of tumors such as osteosarcoma, breast cancer, prostate cancer, and melanoma. In these studies, Notch activity seems to participate in cancer metastasis by modulating the EMT, tumor angiogenesis processes, and the anoikis-resistance of tumor cells. Therefore, manipulating Notch signaling may represent a promising alternative/complement therapeutic strategy targeting cancer metastasis besides cancer stemness.

INTRODUCTION

Cancer metastasis accounts for 90% of deaths of cancer patients. Recent insights have proposed that cancer metastasis, starting from a primary epithelial neoplastic lesion, may

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include: (1) epithelial-mesenchymal transition (EMT) and dissemination; (2) invasion and cell migration; (3) resistance to apoptosis and anoikis; (4) angiogenesis and lymphangiogenesis; (5) intravasation-transport through vessels-extravasation; and (6) outgrowth of secondary tumors.[1,2] Researchers have yet suggested that before the first cancer cell arrives at the distinct organ, the target site has created a "premetastasis niche" for cell colonizing.[3] However, the cellular and molecular mechanisms underlying cancer metastasis and the formation of the premetastasis niche have not been established. Studies on cancer metastasis mechanism may propose possibilities to therapeutically interfere with signaling pathways required for each step of cancer metastasis, therefore inhibiting the outgrowth of distant metastasis of tumors.

CRITICAL CELLULAR AND MOLECULAR EVENTS IN CANCER METASTASIS

EMT and Cancer Cell Dissemination

EMT is a highly conserved cellular program, governing a process by which differentiated, polarized, immotile epithelial cells could be transformed into motile mesenchymal cells.[4] EMT is a vital process for many morphogenetic events during embryonic development and it can also be reactivated in a variety of diseases including cancer.

A hallmark of tumor progression during the invasive and metastatic phases is epithelial cell plasticity and dedifferentiation. Some epithelial-specific proteins, like E-cadherin, occludin, claudins, cytokeratins and catenin, were down-regulated in cancer cells with the metastatic trend.[5] Among them, the cadherin superfamily of Ca^{2+}-dependent homophilic adhesion molecules participates in the regulation of cell-cell interactions during carcinogenesis. For example, the loss of E-cadherin, one member of the cadherin superfamily, is related to the induction of EMT and is observed in the most of aggressive tumors.[6]

Invasion and Cell Migration

Invasive cancer cells can migrate to neighboring tissues or distant organs either as single cells or collectively in the form of strands, files, clusters or even sheets. In the most of tumors, both individual cells and collectives are simultaneously present. However, some tumors such as leukemia, lymphoma and many of solid stromal tumors, migrate via individual cells.[7] Depending on cell types, single cell dissemination could occur in different morphological variants, including mesenchymal and amoeboid types, as well as chains of single cells.[8]

Collective migration of tumor cells is commonly observed in invasive epithelial tumors, such as breast cancer, colon carcinoma and oral squamous cell carcinoma.[9,10] The cell collectives moving as one functional unit require not only cell-cell adhesion, but also signal communication among cells. These characteristics offer advantages on collective migration, by producing autocrine factors and matrix proteases and protecting inner tumor cells from immunological assault.[7]

Many adhesion molecules have been involved in tumor cell migration. Among them, CD44, which mediates cell binding to endothelial venules, is involved in the spread of various malignant cancer cells and its certain splice variants become a landmark of metastasis tumors.[11] The mechanism of tumor cell extravasation from vessels shares many similarities with tumor cell intravasation.[12] For example, TGF-β signaling activation in breast cancer cells could help to disrupt vascular endothelial cell-cell junctions, facilitating

the migration of cancer cells into lung parenchyma. Integrins also play an important role in cell attachment under blood flow conditions.[13]

Resistances to Apoptosis and Anoikis

Anoikis is referred to cell death induced by inappropriate or loss of cell adhesion. It was first identified by Frisch and Meredith who found that both normal epithelial cells and endothelial cells would rapidly undergo programmed cell death, when the interaction between cell and extracellular matrix was interrupted.[14] This process, maintaining the appropriate number of high turnover epithelial cell, is also implicated as a barrier against cancer metastasis by triggering apoptosis. Therefore, dissemination tumor cells have to be resistant to apoptosis in order to move, reattach and colonize to distant organs successfully.[15]

It has been identified that integrins can suppress anoikis by activating focal adhesion kinase (FAK).[16] Integrins can also cooperate with various oncogenic events such as the down-regulation of E-cadherin to promote anoikis-resistance in cancer cells.[17] Besides the suppression of anoikis by cell-cell adhesion interference, interruption of the apoptotic machinery may also contribute to the anoikis suppression.[18]

Angiogenesis and Lymphangiogenesis

The continued tumor growth is often associated with neovascularization.[19] Intratumoral hypoxia up-regulates the expression of the vascular endothelial growth factor (VEGF) which induces angiogenesis, offering the necessary routes for cell dissemination, changing vascular integrity and permeability and even promoting intravasation and extravasation.[20] Meanwhile, hypoxia selects a subpopulation of tumor cells with an invasive and metastatic phenotype that have the capacities of escaping from the primary tumors.[21]

Lymphangiogenesis is also considered as a potential facilitator of cancer metastasis. Cancer cells move to the regional lymph nodes draining the primary tumor site much earlier than to the distant organs.[22] Cancer cells secrete VEGF to promote the growth of lymphatic vasculature. Increasing lymphatic vessels in tumor tissues promote tumor cells to migrate to local lymph nodes and even to distant organs.[23]

The "Seed and Soil Hypothesis"

The migration of disseminated cancer cells is not in a random pattern. Indeed, metastatic cells usually show a strong preference to migrate to specific organs. For example, breast cancer cells preferentially move to the bone and brain, but not the spleen. Therefore, the "seed and soil hypothesis" first proposed by Stephen Paget, an English surgeon, is formed to explain the nonrandom pattern of cancer metastasis. In this hypothesis, the determination of sites for a secondary tumor formation does not only depend on caner cells equating to the "seed" but also largely being influenced by the characteristics of target organs equating to the "soil". Therefore, cancer metastasis forms only when the nature of target organs is compatible with the requirements of disseminated cancer cells.[24,25]

Metastatic Cancer Stem Cells (CSCs)

Although the relationship between CSCs and metastasis is not elucidated clearly, it has been demonstrated that the number of metastatic cancer colonies is correlated with

the frequency of CSCs in primary tumors. Since 1994, CSCs have been first identified in leukemia and subsequently in various solid tumors.[26-29] The hypothesis of CSCs proposed that CSCs, on the top of a hierarchy in all cell lineages in tumors, could keep self-renewal and differentiate into downstream tumor cell types.

On the other side, the CSC subpopulation displays a higher potential for invasiveness than the subpopulations of nonstem tumor cells.[30] Cancer cells undergone EMT displayed some stem-like properties, such as forming mammospheres in breast cancers and the expression of stem cell markers, indicating that EMT was able to endow breast epithelial cells with stem-like properties.[31] In addition, a subpopulation of $CD133^{+}CXCR4^{+}$ CSCs identified in the invasive front of pancreatic tumors, were able to determine the metastatic phenotype of tumors.[32] Recent research works show that Bmi1, a member of the Polycomb group (PcG) family, plays a critical role not only in the self-renewal CSCs, but also in the metastasis of prostate and breast cancers.[33] The function of PcG proteins in the metastasis and self-renewal of CSCs may depend on its epigenetic silencing of target genes. And the detailed mechanism of their function should be explored in the future.

THE NOTCH SIGNALING PATHWAY

The Notch signaling pathway has been recognized as one of a few signaling pathways that are iteratively used in an enormous diversity of developmental processes. However, in recent decades, the function and dysfunction of this pathway are implicated in multiple aspects of cancer biology, including cancer initiation and metastasis.

The Components of the Notch Signaling Pathway

The core Notch signaling pathway comprises receptors, ligands, transcriptional complex components in the nucleus and downstream genes, which are widely conserved throughout the metazoans. The Notch receptor gene was first cloned in *Drosophila* in 1985, which encodes a large single-pass Type I transmembrane receptor.[34] Mammals have four Notch receptors, which have the extracellular domain (ECD) containing tandem epidermal growth factor (EGF)-like repeats mediating interactions with ligands, a transmembrane domain (TMD) and the intracellular domain (NICD) composed of a RAM (RBP-J association molecule), nuclear localization signals (NLS), a ankyrin repeats (ANK), transactivation domain (TAD) and a PEST region involved in protein degradation.[35]

The two major classes of Notch ligands in *Drosophila* are Delta and Serrate, while they give rise to five ligands in mammals as Delta-like (Dll)1/3/4 and Jagged1/2, respectively. Like Notch receptors, all of the ligands are single-pass Type I transmembrane proteins, with a specific DSL domain as a putative Notch-binding surface.[35]

The signal-induced transcriptional activation complex mainly comprises the DNA-binding protein RBP-J (also termed CBF1) and the Mastermind-like (MAML) protein.[35] This protein complex, in turn, directs the assembly of additional co-activators that drive target gene expression. Although RBP-J has been generally accepted as the major effector of Notch pathway, RBP-J-independent noncanonical Notch signaling have also been reported.[36]

In spite of numerous RBP-J binding sites throughout the genome, until now, only the basic helix–loop–helix (bHLH) transcriptional repressors, for example, the hairy and

enhancer of split (HES) family genes have been identified as canonical downstream effector genes.[37] In addition, some tissue specific downstream genes have been uncovered, such as Myc oncogene regulated by Notch in T-lymphocytes.[38] Concerning the pleiotropic effects of Notch pathway, the whole spectrum of Notch transcriptional targets in genome has yet to be discovered.

The Regulation of the Notch Signaling Pathway

The regulation of the Notch signaling pathway seems considerably complicated, with a growing roster of regulatory molecules been found. Productive Notch ligand-receptor binding depends on posttranslational modifications, such as glycosylation of receptors mediated by OFUT-1 and Fringe.[39] And the half-time of Notch and DSL proteins on membrane are determined by the endocytosis of receptors and ligands, executed mainly by ubiquitin E3 ligase such as Deltex and Mindbomb, respectively. Mutations that stabilize NICD can cause T-cell acute lymphoblastic leukemia in humans.[37] On the other hand, the local distribution of Notch receptors on the cell membrane are controlled by some polarity proteins, for examples, Numb and Crumbs, which results in region-specific Notch activity.[37,40]

On the binding of Notch ligands, Notch receptors are cleaved by the presenilin complex that has a γ-secretase activity, releasing NICD. NICD then translocates into the nucleus. Like Notch receptors, Notch ligands as Type I transmembrane protein are also subject to transmembrane domain cleavage by γ-secretase. Ligand processing may be important for its down-regulation and membrane clearance. Alternatively, it could generate biologically soluble ligands that may acts as antagonists of Notch signaling.[37,40]

In the absence of NICD, the DNA-binding protein RBP-J associates with corepressors (CoRs), such as MINT and histone deacetylases (HDACs) to repress its target promoters.[35,37] The binding of NICD to RBP-J facilitates displacement of transcriptional repressors. The NICD/RBP-J interface is then recognized by MAML and this ternary complex recruits coactivators (CoAs) such as histone acetyltransferases (HATs) and chromatin-remodeling factors, to assemble an active transcriptional complex on target promoters.[35,37,40]

NOTCH SIGNALING PATHWAY AND CANCER METASTASIS

Notch signaling has long been implicated in cancer biology. Both of Notch receptors and ligands are transmembrane proteins, therefore signaling is activated upon adjacent cell interaction which is very important for the metastasis process depending on cell-cell interaction and adhension. Recently, several independent research works revealed that Notch signaling could regulate tumor cell metastasis in different tumor types (Table 1).

Osteosarcoma

Osteosarcoma is the most common cancer in bone tissues with 10-year disease free survival rate no more than 30%. The primary osteosarcoma is susceptible to metastasize to the lung, with the majority of patients presenting pulmonary micrometastases.[41]

Notch signaling pathway and its components play a critical role in patterning the mammalian axial skeleton.[42] Over-activation of Notch impairs osteogenesis and enhances adipogenesis in stromal cell cultures.[43] The activation of Notch signaling was also

Table1. Notch signaling and cancer metastasis

Type of Cancer	Aberrant Notch Signaling Component	Effect on Cancer Metastasis	Refs.
Osteosarcoma	High expression of Hes1	promotion	45
Breast cancer	High expression of Jagged1	promotion	47,48
Prostate cancer	High expression of Jagged1	promotion	52
Melanoma	Activation of Notch1	promotion	56,57
Pancreatic cancer	Activation of Notch1	promotion	58
Gastric cancer	Activation of Notch1	promotion	59
Colorectal cancer	High expression of Hes1	promotion	60

associated with osteosarcoma.[44] Notch pathway components, including Notch1, Notch2, Dll1 and Hes1, were all expressed in osteosarcoma cells. The expression of Hes1 was associated with invasive and metastatic potential of osteosarcoma.[45] The inhibition of Notch signaling pathway with γ-secretase could eliminate the invasion of osteosarcoma cells in Matrigel without affecting cell proliferation, survival, or anchorage-independent growth. In addition, their further research work in mouse model revealed that inhibition of Notch/Hes1 signaling pathway suppresses osteosarcoma metastasis in vivo (Table 1).[45]

Breast Cancer

Recent studies also pointed to a role for Notch signaling in human breast cancer metastasis. All four Notch receptors, four of five Notch ligands and one of the three Fringes have been reported to be expressed in human breast cancer at various combinations.[46] Among them, the expression of Notch ligands, such as Jagged1, is associated with breast cancer invasiveness and metastasis (Table 1).[47] Researches show that elevated levels of Jagged1 correlates with increased expression of Slug.[47] Notch could also up-regulate Snail in endothelial cells to promote mesenchymal transformation (Table 1).[48] Slug and Snail are sufficient to induce EMT and metastasis by repressing the expression of E-cadherin.[49]

Prostate Cancer

The progression of prostate cancer is uncontrollable because of its gradually formed resistance to hormone therapies and cancer metastasis. Martin et al identified several androgen-regulated proteins, including the Notch ligand Jagged1, in prostate cancer cell lines.[50] Previous studies have demonstrated that the activation of Notch signaling could inhibit prostate cancer cell proliferation.[51] Therefore, the increase in the level of Jagged1 mediated by hormone at least may play an important role in the growth and survival of prostate cancer cells. On the other side, Santagata et al described the association between Jagged1 expression and prostate cancer metastasis and recurrence. Compared with benign prostatic tissues or localized prostate cancer, Jagged1 is more highly expressed in metastatic prostate cancer cells, associated with cancer recurrence after radical prostatectomy (Table 1).[52]

Melanoma

The high propensity of melanoma to metastasize and its resistance to chemotherapy and radiation, are responsible for the high mortality rate of this malignant skin cancer.[53] The role of Notch signaling in maintaining normal melanocyte homeostasis remains poorly characterized. However, quite a few literature detailed the similarity of molecular signature between aggressive melanoma cells and human embryonic stem cells (ESCs), in their expression patterns of genes such as Notch receptor, CD133, Wnt-5a and Nodal.[54,55] These studies suggested that the Notch pathway is activated in human melanoma. Blocking Notch signaling suppressed the growth of primary melanoma cells, whereas activation of Notch1 enabled primary melanoma cells to gain metastatic capability (Table 1).[56,57] Furthermore, the up-regulated expression of β-catenin and N-cadherin, followed Notch1 activation, was responsible for the enhanced tumor metastasis.[56,57]

THE MECHANISMS OF NOTCH FUNCTION ON CANCER METASTASIS

Notch signaling influences numerous cellular processes by utilizing different mechanisms. It is instrumental in development by regulating stem cell proliferation, linage decision and boundary formation. In adult animals, Notch signaling has recently been shown to regulate dendritic cell migration by modulate chemokine receptor expression.[61] In terms of tumor cell metastasis, Notch signaling seems to affect the processes of EMT, angiogenesis and anoikis of tumor cells (Fig. 1).

Notch Signaling Converts the Hypoxic Stimulus into EMT

EMT describes the differentiation switch between polarized epithelial cells and motile mesenchymal cells, which facilitates cell movements and the generation of new tissues during both embryogenesis and cancer progression. A great number of studies have shown that EMT contributes to tumor invasion and vascular intravasation during cancer metastasis. The mechanisms of Notch function on cancer metastasis are closely related with EMT.[48,62] Tumor hypoxia is linked to enhanced EMT and increased metastatic potential and Notch signaling is required to convert the hypoxic stimulus into EMT, increased motility and invasiveness.[62] EMT is mediated, in part, by two transcription repressors, Snail and Slug. Sahlgren et al showed that Notch signaling adopts two synergitic mechanisms to control the expression of Snail-1. One is to directly up-regulate Snail-1 expression by recruitment of the NICD to the Snail-1 promoter and the other is to potentiate HIF-1α recruitment to the lysyl oxidase (LOX) promoter and elevate LOX expression, which stabilizes the Snail-1 protein.[62] In addition, Chen et al revealed that hypoxia increased the expression of Notch target genes such as *Hes1* and *Hey1* in breast cancer cells and they further demonstrated that HIF-1α could bind to *Hes1* promoter and enhanced its expression.[48] In both of these studies, Notch pathway inhibition abrogated the hypoxia-mediated increase in Slug and Snail expression, as well as decreased cancer cell migration and invasion.[48,62] Therefore, hypoxia-mediated Notch signaling may have an important role in the initiation of EMT and subsequent potential for cancer metastasis (Fig. 1).

On the other hand, TGF-β signaling is a major inducer of EMT not only during embryonic development, but also during cancer progression in mouse models.[63] TGF-β and Notch signaling converge in the regulation of a number of developmental and tumorigenic

processes. TGF-β increases the expression of *Hes1*, a direct target of Notch, in several cell types. It also induces the interaction of the intracellular domain of Notch1 with Smad3, an intracellular transducer of TGF-β signaling.[64] In addition, Zavadil et al demonstrated that TGF-β signaling can up-regulate the expression of Notch ligands, such as Jagged-1, which activates Notch signaling, leading to EMT and epithelial cell cycle arrest in cell models in vitro.[65] In breast cancer, EMT is correlated with the highly aggressive metastatic spread of these tumors. TGF-β-induced EMT can be blocked by RNA silencing of the Notch target gene Hey1 and the Notch ligand Jagged1 and by chemical inactivation of Notch.[65] In summary, the Jagged1/Notch signaling mediates EMT in cancer metastasis with the integration of the TGF-β signaling.

Besides EMT, the influence of Notch signaling on the adherence junctions (AJs) or matrix metalloproteinases (MMP) has also been shown to affect cancer metastasis. Both of N-cadherin and β-catenin are major components of the AJs structure. Zheng et al have shown that blocking Notch signaling destroyed the AJs between retinal progenitor cells during development.[66] In cancer progression, Liu et al demonstrated that Notch1 signaling promotes primary melanoma invasion by the up-regulation of N-cadherin expression.[57] The N-cadherin and β-catenin-mediated AJs seems to enhance the implantation of metastatic tumor cells into none primary tissues. Accordingly, Balint et al further reported that Notch activity could facilitate the metastasis of melanoma. In their study, the activation of Notch1 signaling could enhance the stability of β-catenin protein in melanoma cell and promote human primary melanoma progression.[56] Although they demonstrated that the stabilized β-catenin mainly maintain the survival of primary melanoma cells, it is also possible that they could affect AJs between melanoma cells and primary tissue cells. In addition, Wang et al reported that Notch signaling plays a critical role in pancreatic cancer cell invasion. Their research showed that the down-regulation of Notch1 reduced nuclear factor-κB (NF-κB) DNA-binding activity and its target genes, such as MMP-9 expression.[58]

Blocking Notch Signaling Produces Dysfunctional Tumor Vessels

Notch signaling molecules have an important well-documented role in vascular development and tumor angiogenesis. Genetic studies in mice with disrupted Notch pathway components display various defects in blood vessel formation.[67] In summary, Notch signaling mainly promotes the development of arteries, determines the choice of tip cell/stalk cell commitment, inhibits the proliferation of vascular endothelial cells and probably participates in the migration and pseudopod formation processes of vascular endothelial cells.[68-71] In adulthood, Notch signaling plays a critical role in the maintenance of homeostasis of normal vasculature by repressing endothelial cell proliferation.[72]

Concerning tumor angiogenesis, since Notch signaling has been shown to maintain the homeostasis of adulthood vasculature, it has been speculated that blocking Notch signaling will lead to angiogenesis in solid tumor. Indeed, several research groups performed general Notch blocking in tumor recipient animal and observed the growth and angiogenesis of solid tumor. Most of them choose *Dll4* as the target, by using anti-*Dll4* antibody, soluble *Dll4*-Fc molecule or *Dll4* RNA interference strategy to block Notch signaling.[73–77] To their surprise, all these studies reached a similar conclusion that although the angiogenesis in solid tumor is greatly enhanced, the growth rate of tumor is slow down. Their further observation found that the hyperplastic vasculature in Notch-blocked tumors with severe morphological impairment and functional deficiency, leading to poor perfusion and enhanced tissue hypoxia in the bulk of tumors.[73-77] However, these dysfunctional tumor

vessels with high permeability are important structure foundation for tumor metastasis.[78,79] Accordingly, Hu et al found that hepatocarcinoma implanted in RBP-J knockout mice lead to increased deregulated angiogenesis and displayed liver metastasis tendency.[80] This result indicates that hyperplastic vasculature with high permeability in RBP-J knockout mice might facilitate the liver metastasis of implanted tumor. In summary, blocking Notch signaling in patients might facilitate tumor metastasis while decrease tumor growth, by promoting dysfunctional angiogenesis (Fig. 1).

Notch Signaling Promotes Anoikis-Resistance of Tumor Cells

Anoikis induced by the loss of cell adhesion is an important barrier against metastasis by triggering apoptosis of disseminating tumor cells. Tumor cells must be resistant to anoikis in order to achieve metastasis. Notch signaling seems to participate in the resistance of anoikis of tumor cells, shown by a few independent research works (Fig. 1). Leong et al reported that in breast cancer, the Notch-mediated repression of E-cadherin resulted in β-catenin activation and resistance to anoikis. In their model, Notch activation facilitates tumor metastasis by the promotion of EMT and the inhibition of anoikis.[47] In addition, Rangarajan et al showed that activated Notch1 synergizes with papilloma virus oncogenes in the transformation of immortalized epithelial cells and leads to their resistance to anoikis, on matrix withdrawal.[81] Their further evidence showed that the resistance to anoikis by activated Notch1 is mediated through the activation of PKB/Akt, a key effector of activated Ras in transformation.[81]

On the other hand, a few research works show that the cell population with anoikis-resistance is probably the enrichment of CSCs. Harrison et al enriched stem cells in breast cancer by selection of anoikis-resistant cells and found that Notch4 signaling activity was 8-fold higher in stem cell-enriched cell populations compared with differentiated cells, linking Notch signaling to CSCs and the resistance of anoikis.[82] In fact, the blockade of Notch pathway depletes CD133-positive glioma stem cells, indicating that Notch signaling participates in the maintenance of at least some type of CSCs (Fig. 1).[83] Further analyses are required to elucidate the function of Notch signaling on the resistance of anoikis and its relation with Notch function on CSCs.

CONCLUSION

The multiple aspects of Notch function in tumor biology making it a potential drug target for cancer therapy. However, it is hard to decide a general strategy to enhance or block Notch signaling in tumor progression concerning with the complicated biological effects of Notch function in different pathological contexts of various tumor types. In tumor metastasis, Notch signaling seems to facilitate it by promoting EMT and the resistance of tumor cells to anoikis and apoptosis. On the other hand, blocking Notch signaling results in hyperplastic vasculature with high permeability, by which metastasis more easily occurs (Fig. 1). In addition, the intervention of Notch signaling in tumor progression will not only affect metastasis, but meanwhile alter the tumor growth, the infiltration of immunocells such as tumor-associated macrophages (TAMs)[84] and dendritic cells (DCs) and the maintenance of CSCs. In summary, the Notch function in tumor biology could be a multi-edged sword and the utilization of it as a target of cancer therapy should be considerably prudent, depending on extremely rigorous preclinical experiments in specific cancer types.

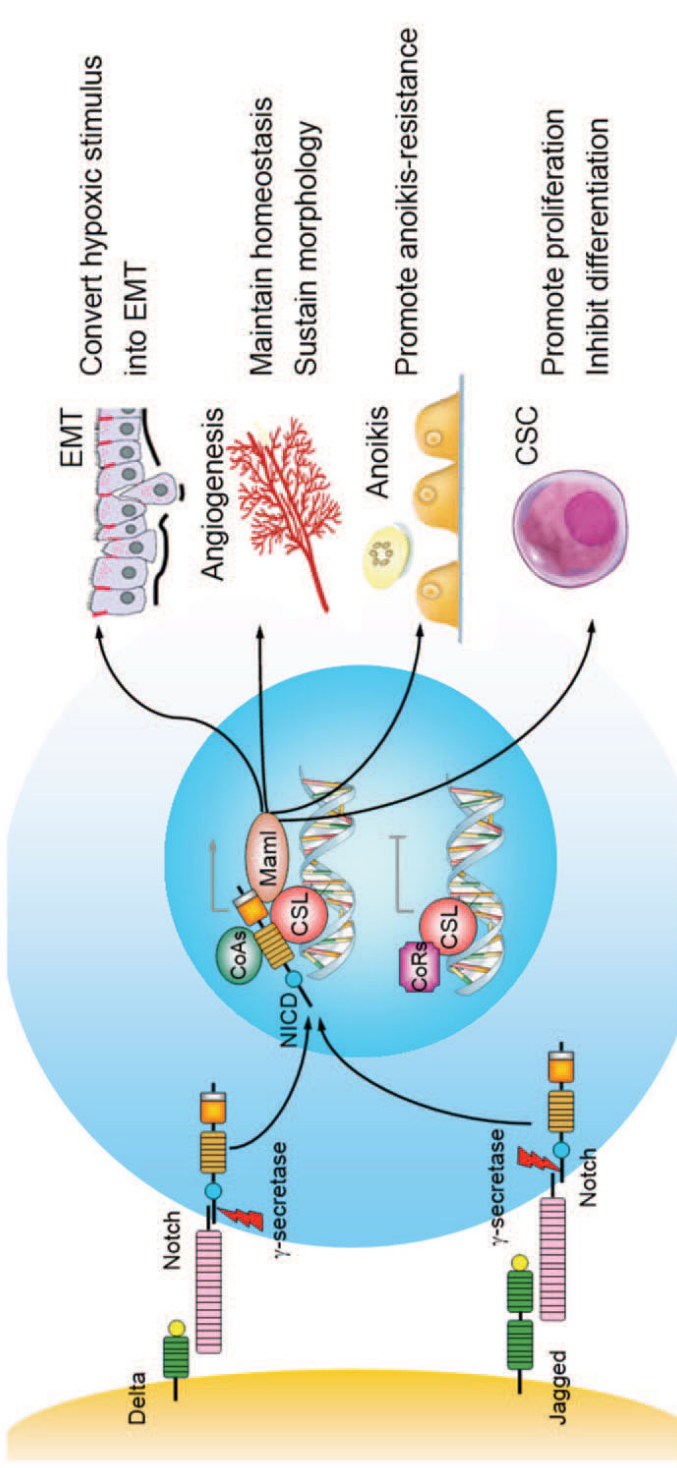


Figure 1. The Notch signaling pathway and its roles in cancer metastasis. The Notch receptors are activated by the Delta-like and Jagged families of ligands expressed on adjacent cells. Upon γ-secretase-mediated proteolysis, NICD proteins translocate to the nucleus and bind to the DNA binding protein CSL, taking the place of the CoRs. NICD forms a complex with the DNA binding protein CSL and CoAs, leading to the transcriptional activation of Notch target genes. The activation of Notch signaling in tumor microenvironment could promote EMT, the anoikis-resistance of tumor cells and maintain the homeostasis of angiogenesis, the morphology of vasculatures and the self-renewal of CSCs.

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CHAPTER 15

NOTCH, APOPTOSIS AND CANCER

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Abstract: Proper embryonic development and normal tissue homeostasis require a series of molecular processes, regulating cell growth, differentiation and apoptosis. Perturbation in any of these processes invariably contributes to the development of cancer. In particular, defects in apoptosis are seen in virtually all types of human cancers. The Notch pathway plays an important role in cell fate determination in both embryonic development and organ homeostasis. Not surprisingly, Notch also plays a role in cancer when it is dysregulated. In this chapter, we will explore how Notch signaling interacts with key pathways that regulate apoptosis in cancer. Particularly, we will focus on the relationship between Notch and proteins responsible for activation of the caspase pathway. Notch regulates apoptosis through extensive networks, involving cell cycle, growth and survival pathways. Thus, we will also examine how apoptosis is modulated by the crosstalk between Notch and other signaling pathways such as p53, NF-κB and PI3K-Akt pathways.

INTRODUCTION

Apoptosis, often referred to as programmed cell death, is an important process in virtually all multi-cellular organisms, directing proper organogenesis during development and maintaining tissue homostasis. For example, during the development of the limb, apoptosis selectively eliminates extraneous cells in the interdigital mesenchymal tissue to allow formation of independent digits. It is also responsible for the destruction of ineffective T-cells during maturation of the immune system.[1] Moreover, apoptosis serves as a mechanism of cellular defense against external insults, such as ionizing radiation, by eliminating cells that were irreversibly damaged.

Activation of the cellular proteases known as caspases is central to initiating programmed cell death.[2] There are two, major pathways responsible for initiating the

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caspase cascade. The first pathway involves a subgroup of the tumor necrosis factor receptors (TNF-R) and includes the TNF-R1 and the Fas receptor. This pathway is also referred as the "extrinsic" or "death receptor" pathway. The second pathway, known as the "intrinsic" or "mitochrondrial" pathway, is regulated mainly by the Bcl-2 family of proteins. In mammals, the Bcl-2 family includes both pro-apoptotic as well as anti-apoptotic proteins in which the ratio between the two groups determines the cell's susceptibility to apoptosis. Unlike the "death receptors" pathway, the hallmark of the "intrinsic" pathway is that mitochrondia are involved in the activation of caspases and the amplification of external death signals.

Morphologically, the apoptotic cells undergo a series of changes that included membrane blebbing, chromatin condensation and DNA fragmentation. A defect in the apoptotic machinery can contribute to cancer, autoimmune diseases and degenerative diseases, depending on whether the cells fail to undergo programmed cell death, or whether they die prematurely.[3-5] As we discuss in detail below, Notch regulation of apoptosis is multifactorial, involving complex crosstalks with many pathways that regulate cell cycle, growth and survival.

Notch signaling was first discovered over 90 years ago, when a heterozygous deletion resulted in "notches" at Drosophila wing margins.[6] In 1985, the genetic sequence of Drosophila Notch was published, giving greater insight into its structure and function.[7] Drosophila Notch is a transmembrane protein containing 36 repeats with homology to the 40 amino-acid repeats that are seen in the epidermal growth factor (EGF) receptors and other proteins containing EGF-like repeats. The initial studies of this pathway mainly focus on Drosophila, where Notch was found to be important for normal development of many tissues, such as eyes and wings.[8] Since its original discovery, a role for Notch signaling in cell-fate determination and development has been established in virtually all multi-celluar organisms, including mammals.

Notch receptors and their ligands are Type I, transmembrane proteins. In mammals, there are four Notch receptors (Notch1 to Notch4) and two families of ligands, Jagged (Jagged1, 2) and Delta-like (Dll1, -3, -4). More extensive reviews of Notch signaling and processing are covered in the previous chapters (or elsewhere[9]) and will not be elaborated in detail. Briefly, the binding of membrane-bound Notch ligand to the Notch receptor results in two successive proteolytic cleavages by an ADAM-type metalloprotease and by a γ-secretase, respectively. The Notch intracellular domain (NICD), now untethered from the cytoplasmic membrane, is then translocated to the nucleus. Upon binding to CSL (CBF1, Sel, Lag-1), also known in mammals as RBP-Jκ, NICD converts CSL from a suppressor to a transcriptional activator and induces expression of target genes, among which are genes belonging to the *Hairy-enhancer of Split (HES)* and the *hairy and Enhancer-of-split related with YRPW motif (Hey)* gene families.

Because growth, differentiation and apoptosis are commonly aberrant in cancer development, genes such as the *Notch* gene play a role in tumorigenesis when they are dysregulated. Indeed, aberrant Notch signaling is associated with malignant phenotypes in both hematologic and epithelial cancers, and all four members of the Notch family play some role in human cancers.[10-13] Suppression of Notch signaling results in the loss of the malignant phenotype both in vitro and in vivo models.[13,14] Furthermore, Notch has been shown to cooperates with other oncogenic pathways in maintaining tumor phenotypes, further supporting the role of this pathway in tumorigenicity.[15,16]

p53 PATHWAY

p53 functions as a cell-cycle regulator, by inhibiting cell cycle progression and promoting apoptosis after DNA damage. When a cell is exposed to DNA damaging agents, e.g., chemotherapeutics, UV-light or ionizing radiation, p53 expression is upregulated, resulting in activation of p21 and eventual cell cycle arrest. At this juncture, the damaged cell is targeted either for repair or for apoptosis, depending or whether the damage is reversible.[17] The p53 pathway is often dysregulated in cancer. In fact, inactivating mutations of the p53 gene are observed in over 50% of all human cancers.[18] p53 is inactivated by murine double minute 2 (Mdm2) through its E3 ubiquitin-ligase activity, and its half-life is prolonged when the Mdm2 is sequestered by INK4a/ARF. About 5% of cancers shows amplification of the Mdm2 locus, while *INK4a/ARF* is inactivated in about 50% of cancers.[19-21]

Because of the Notch pathway's impact on apoptosis and cancer, it is not surprising that Notch crosstalks with the p53 pathway. Expression of activated Notch1 is often negatively correlated with that of p53. Current data suggest that the Notch pathway can suppress p53 expression and activity through a variety of mechanisms. One mechanism includes the repression of INK4a/ARF, resulting in unopposed ubiquitination of p53 by Mdm2[22] (Fig. 1). In colorectal cancer, Notch1 can inactivate exogenous p53 by inhibiting its phosphorylation.[23] While many studies support the role of Notch in downregulating p53 mainly through enhanced degradation, some have shown that RBP-Jκ, a Notch-related transcription factor, can bind to the p53 promoter and repress its transcription.[24]

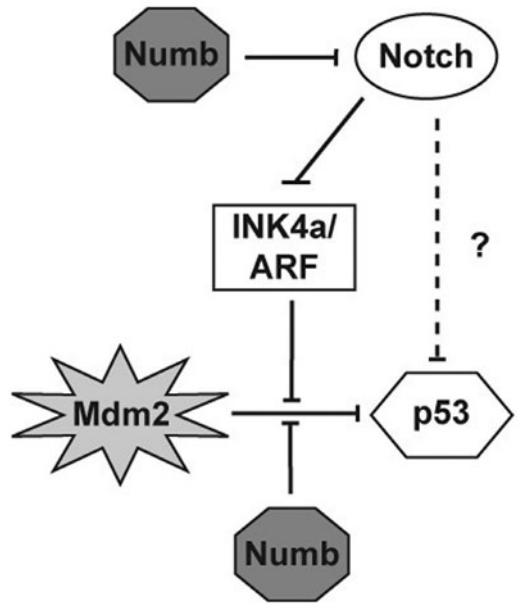


Figure 1. Notch inhibits the p53 pathway in apoptosis. Activated Notch represses the expression of tumor suppressor INK4a/ARF, resulting in the unopposed degradation of p53 by Mdm2. Numb, an inhibitor of Notch signaling, binds to the Mdm2-p53 complex and prevents degradation of p53. Notch can suppress transcription of p53 through the binding of the p53 promoter by RBP-Jκ.

Numb is an adaptor protein that was first identified as a Notch inhibitor. Numb sequest activated Notch in the cytoplasm and complexes it to ubiquitin ligases such as Itch and LNX, resulting in enhanced degradation of Notch protein.[25] Like many tumor suppressor genes, *Numb* is downregulated in about 30-50% of epithelial tumors.[26,27] Recent studies provide evidence for a role of Numb in regulating other developmental pathways such as the Hedgehog pathway. While Numb is a negative regulator of Notch and Hedgehog, it enhances p53 function by binding to the Mdm2-p53 complex, preventing ubiquitination and degradation of p53. The binding site appears to be distinct from that of the Mdm2-p53 interaction, suggesting that a tri-complex is formed in vivo.[28] How the tri-complex is formed remains unclear, because Numb is located predominantly in the cytoplasm and the endosomes, whereas the Mdm2-p53 complex is located in the nucleus.

While the majority of data suggest that Notch suppresses p53 functions in cancer, overexpression of activated Notch has been shown to sensitize tumors to death signals in a p53-dependent manner.[29,30] Furthermore, restoration of p53 in tumors can result in the upregulation of activated Notch.[31,32] Whether Notch functions as a tumor suppressor in this context or as a negative feedback response will need further elucidation. Global mapping of the p53 transcription-factor binding sites identified Notch1 as a target of p53, suggesting that transcription represents a potential mechanism of p53-dependent Notch1 induction.[33] Thus, like many other signaling pathways in a biological system, the phenotypic outcome of Notch activity in regard to apoptosis is highly context dependent.

NF-κB PATHWAY AND NOTCH IN APOPTOSIS

The nuclear factor κB (NF-κB) pathway plays a diverse role in numerous cell functions, including immunity, proliferation, inflammation and apoptosis. In the context of cancer, this pathway contributes to all aspects of oncogenesis, such as survival, invasion, proliferation and angiogenesis (for a review of NF-κB signaling see[34]). In mammals, this family comprises of five members, class I (NF-κB1 (p50 and its precursor p105) and κB2 (p52 and its precursor p100)) and class II (RelA (p65), RelB, cRel). These proteins form homodimers or heterodimers. They are regulated by two major pathways. In the classical or canonical pathway, the NF-κB dimers are sequestered in the cytoplasm through their interaction with IκB and remain inactive. Through various stimuli, such as tumor necrosis factor α (TNFα), interleukin 1 (IL-1), viruses, or DNA damaging agents, an IκB kinase (IKK) complex is induced, resulting in the phosphorylation and ubiquitiniation of IκB. Without the inhibitory effect of IκB, the NF-κB heterodimers are now free and translocate to the nucleus to induce the transcription of target genes. The second or noncanonical pathway involves NF-κB-inducing kinase (NIK), which activates IKKα, leading to proteosome-dependent processing of p100 (the precursor to p52). This pathway is activated by cytokines belonging to the TNF superfamily.

The most well-known function of NF-κB includes its ability to promote cell survival and inhibition of this pathway often leads to apoptosis.[35,36] A considerable body of studies conducted over the last decade has demonstrated that the effect of Notch signaling on apoptosis occurs through its regulation of the NF-κB pathway (Fig. 2). Oswald et al first described binding of RBP-Jκ the promoter element within the *NF-κB2* gene. RBP-Jκ normally represses transcription of NF-κB precursor p100. However, in the presence of activated Notch, the promoter activity is induced, leading to increased transcription.[37] In addition, Notch regulates expression of other NF-κB subunits such as p50, RelA, RelB

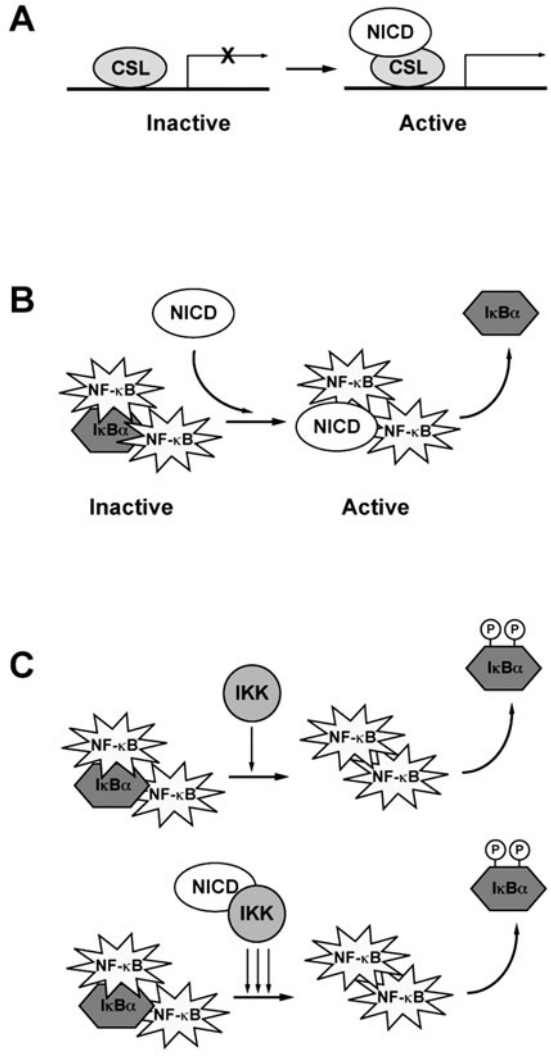


Figure 2. Proposed models of how Notch modulates the NF-κB pathway in regulating apoptosis, A) Activated Notch induces transcription of NF-κB genes p50, RelA, RelB and cRel. RBP-Jκ, a Notch CSL protein, normally represses transcription. In the presence of Notch intracellular domain (NICD), transcription is activated. B) NICD competes with IκBα for NF-κB binding. The interaction with NICD releases NF-κB dimers from inhibition by IκBα and results in their activation. C) In another model, Notch enhances NF-κB activity by binding to IKK and promotes degradation of IκBα.

and cRel.[38] Interestingly, RBP-Jκ also represses transcription of IκBα, and in the presence of Notch ICD the transcription is similarly reactivated.[39] While Notch upregulates the expression of NF-κB subunits, activation of NF-κB induces the expression of the Notch related proteins, such as the ligand Jagged1, Hes-5 and Deltex-1.[40,41] Thus, Notch and NF-κB pathways interact through transcriptional regulation both positively and negatively depending on the cellular context.

The activated domain of Notch1 (ICD) was noted to contain six ankyrin repeats that bear similar homology to the cytoplasmic IκB protein. Subsequent experiments demonstrated that the ICD also binds to p50-containing NF-κB complexes.[42,43] While the initial studies suggested that the Notch1-NF-κB interaction results in the transcriptional suppression of NF-κB target genes, other investigators have shown more recently that Notch competes with IκB for binding with p50 complexes and enhances NF-κB activities.[44] In a murine model of T-cell acute leukemia (T-ALL), Vilimas et al observed that Notch1 ICD also interacts with IKK complexes, enhancing IκB phosphorylation. The resulting constitutive NF-κB activation appears to be important for the survival of leukemic cells.[45] As Notch promotes tumor survival through its regulation of IKK, IKKα in turn promotes Notch activity through phosphorylation of the nuclear receptor corepressors SMRT (silencing mediator of retinoid and thyroid receptors), allowing Notch-dependent transcription of target genes to proceed.[46]

PI3K-Akt PATHWAY

A hallmark of virtually all types of cancer is the ability of cancer cells to survive without exogenous growth factors. The majority of these growth signals are the receptor tyrosine kinases (RTKs). A target for these RTKs is the phosphoinositide 3-kinase (PI3K) pathway, known to promote tumor survival and resistance to apoptosis from chemotherapy. The recruitment of PI3K by RTKs to the membrane results in the phosphorylation of phosphoinositol lipids from phosphoinositide- 4,5 biphosphate (PIP2) to phosphoinositide-3,4,5 trisphosphate (PIP3). These specialized lipids recruit serine-threonine kinase Akt to the plasma membrane, where Akt is phosphorylated and subsequently activated. Akt, in turn, phosphorylates multiple target genes, resulting in the inhibition of the pro- apoptotic proteins BAD and caspase 9 and in the activation of the pro-survival proteins IKK and mTOR (for a review see ref. 47). PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a phosphatase that converts PIP3 back to PIP2 and serves as a negative regulator of the PI3K-Akt pathway. PTEN is frequently downregulated and mutated in many types of cancer and the loss of PTEN activity is thought to be a major mechanism of drug resistance.[48]

The mechanism of Notch-dependent oncogenic transformation is complex and multifactorial, but it often involves the cooperation with the growth factor pathways discussed above.[49,50] Inhibition of apoptosis in Notch-transformed tumors and resistance to chemotherapy appear to be dependent on PI3K-Akt pathway, particularly breast cancer.[51,52] Similarly, under hypoxic conditions, Notch1-dependent survival of cancer cells is abolished by Akt inhibition.[53] While further studies are needed to better understand the crosstalk between the Notch and the PI3K-Akt pathways, current evidence suggests that Notch does not directly affect PI3K or Akt. In T-cell acute leukemias (T-ALL), where nearly 75% of cancer cell lines do not have detectable PTEN expression, the Notch-targeted protein HES1 represses transcription of PTEN.[54] The same investigators also showed that homozygous PTEN mutations represent a mechanism of acquired resistance to apoptosis induced by γ-secretase inhibitors. Interestingly, some investigators have shown that nuclear localization of Notch ICD and HES1 transcription are not necessary to inhibit Bax-dependent apoptosis, supporting a noncanonical pathway in its crosstalk with the PI3K/Akt pathway.[55] Others demonstrated that Notch1 modulates Akt activity through transcription of *insulin-like growth factor 1 receptor* (IGF-1R), suggesting that Notch

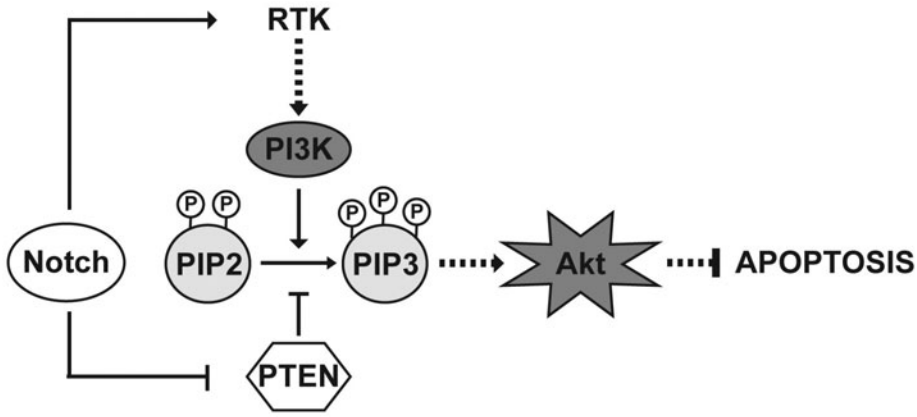


Figure 3. Notch crosstalks with the PI3K/Akt pathway in regulating apoptosis. Available data indicate that Notch modulates Akt activity through transcriptional repression of PTEN and upregulation of receptor tyrosine kinase, e.g., IGF-1R.

regulation also occurs further upstream.[56] Moreover, these authors showed that *IGF-1R* transcription is independent of HES1. Instead, Notch ICD and its co-activator MAML-1 regulate *IGF-1R* transcription by binding directly to the +1478 DNA region of the *IGF-1R* gene. Taken together, these data suggests that Notch utilizes and depends on the PI3K/Akt pathway in inhibiting apoptosis in certain context. Furthermore, this mechanism involves the transcriptional regulation of PI3K/Akt related genes, possibly independent of the canonical pathway (Fig. 3).

NOTCH AND THE MAJOR APOPTOTIC PATHWAYS

Like Notch, the Bcl-2 protein family members play an important role in both development and cancer.[57,58] They are the central components of the "instrinsic" or "mitochrondria" pathway and regulate programmed cell death through the integration of diverse extra- and intracellular death signals. There are three subfamilies in the Bcl-2 family of proteins. Structurally, the pro-survival proteins Bcl-2, Bcl-xL and Mcl-1 and the pro-apoptotic proteins Bax and Bak contain between 2-4 characteristic motifs (BH1-4; Bcl-2 Homology domains). Whether the cell is targeted for programmed cell death is determined by the tightly regulated balance between these two groups of proteins. The third group consists of BH3-only proteins, which share homology with the other Bcl-2 family members only in the BH3 region. They function mainly as "sensors" or initiators of apoptosis.[59] They can be activated by DNA damage, in the case of Noxa and Puma, or by growth factor deprivation in the case of Hrk and Bim. The BH3-only proteins promote apoptosis by inhibiting anti-apoptotic members like Bcl-2, thus allowing Bax and Bak to permeabilize the mitochrondrial outer membrane, resulting in cytochrome *c* release and ultimately activating the caspase cascade.

The Notch pathway has been shown to promote expression of pro-survival proteins Bcl-2 and Bcl-xL and to attenuate the expression of the pro-apoptotic proteins Bax and Bim.[14] Particularly, constitutive Notch3 represses Bax mRNA levels, while inducing

Bcl-xL transcription.[60] Currently, there is no evidence for a direct link between Notch and the Bcl-2 families members. Rather, the data suggest that Notch exerts control over these proteins transcriptionally and through other pathways such as NF-κB or the PI3K/Akt pathways.[61,62] In lung cancer, Bim has been shown to be necessary for the induction of apoptosis by Notch3 inhibition. While the Notch3 and the ras/MAPK pathway cooperate in maintaining malignant phenotype, the regulation of Bim by Notch3 appears to depend on the MAPK pathway.[14,13] In myeloma and melanoma, inhibition of Notch signaling induces the expression of Noxa through HES1 transcription. While Noxa is known to be a p53 target protein, this regulation appears to be p53-independent.[63,64]

The interaction of the Notch pathway with the "extrinsic" or "death receptors" pathway is not well studied. As mentioned previously, the "death receptors" are cell surface proteins and belong to a tumor necrosis factor receptor (TNF) family, which includes Fas/CD95 and the TRAIL receptors. Ligand binding induces trimerization of the receptors. Activation of of the cytoplasmic death domain (DD) results in the recruitment of adapter molecules FADD or TRADD. These adapter molecules, in turn, recruit procaspase-8 and -10, forming the so-called death inducing signaling complex (DISC). The DISC mediates autocatalytic processing of caspases 8 and 10 and releases active enzymes into the cytoplasm, which initiate programmed cell death.[65]

Some evidence supports a crosstalk between Notch and the death receptors pathway. Notch2 is upregulated by death ligands in MCF cells and endothelial cells.[66,67] Conversely, activated Notch3 can attenuate the loss of vascular smooth vessel cells by inducing expression of c-FLIP, a primary inhibitor of the FasL pathway.[68] In T-cells, activated Notch can inhibit Fas-induced apoptosis, albeit indirectly through inducing the expression of Bcl-xL and phospho-Akt.[69]

Another group of proteins that play and important role in regulating apoptosis consists of the inhibitors of apoptosis (IAPs). These proteins, which include the X-linked inhibitor of apoptosis protein (XIAP) and the cellular inhibitor of apoptosis protein 2 (c-IAP2), contain the characteristic baculovirus IAP repeats (BIR). While these proteins exhibit a wide variety of functions, one major role is their ability to inhibit caspases activation.[70] Rosati et al have shown that the Notch pathway promotes B-CLL cell survival through induction of c-IAP2 and XIAP expression.[71] The mechanism of this regulation still needs further elucidation, but Notch ICD was found to inhibit XIAP degradation by binding to the RING domain of XIAP and preventing E2 ubiquitination.[72] These findings suggest that Notch inhibits apoptosis, in part, by prolonging the half-life of the apoptosis inhibitors XIAP and c-IAP2.

CONCLUSION

While Notch was discovered nearly a century ago, only recently have we begun to appreciate its effect on growth, differentiation and cell survival. Programmed cell death, or apoptosis, is a complex process– a balance of pro-apoptotic and pro-survival proteins. This balance is tuned by apoptosis inhibitors (IAPs) and inducers (BH3-only members of the Bcl-2 family). Signals from other pathways such as the p53, NF-κB, PI3K/Akt and MAPK pathways contribute further to the balance, influencing the final outcome.

The role of Notch signaling in apoptosis is heavily context dependent and involves multiple crosstalks with other pathways, not unlike any other complex signaling pathways. In the context of cancer, current data support a strong role for Notch signaling

in maintaining the oncogenic phenotype. Notch appears to regulate apoptosis through extensive networks, involving cell cycle, growth and survival pathways. However, the mechanisms of these crosstalks are far from clear and further investigation is needed to define the interactions, both functionally and structurally. Moreover, whether Notch depends on its canonical pathway through transcription of HES related genes for the crosstalk still needs further elucidation. Cancer remains the second most common cause of death, accounting for about 23% of all deaths in the United States in 2006.[73] Given its role in preventing apoptosis, Notch therefore represents a reasonable target for drug design and intervention. The goal can only be accomplished through a better understanding of the biology of Notch signaling in both cancer and development.

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CHAPTER 17

NOTCH AND THE p53 CLAN OF TRANSCRIPTION FACTORS

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Abstract: Notch 1 to 4 and the p53 clan, comprising p53, p63 and p73 plus numerous isoforms thereof, are gene transcription regulators that are critically involved in various aspects of cell differentiation, stem cell maintenance and tumour suppression. It is thus perhaps no surprise that extensive crosstalk between the Notch and p53 pathways is implemented during these processes. Typically, Notch together with p53 and even more so with transactivation competent p63 or p73, drives differentiation, whereas Notch combined with transactivation impaired p63 or p73 helps maintain undifferentiated stem cell compartments. With regard to cancer, it seems that Notch acts as a tumour suppressor in cellular contexts where Notch signalling supports p53 activation and both together can bring on its way an anti-proliferative programme of differentiation, senescence or apoptosis. In contrast, Notch often acts as an oncoprotein in contexts where it suppresses p53 activation and activity and where differentiation is unwanted. It is no accident that the latter pathways—the inhibition by Notch of p53 and differentiation—are operative in somatic stem cells as well as in tumour cells.

INTRODUCTION

The Notch and p53/p63/p73 proteins (the p53 clan hereafter) all function typically as receptors/sensors-that-turn-into-transcriptional-regulators-upon-stimulus, with the main difference being that Notch senses signals at membrane surfaces through contact with membrane anchored ligands[1] while the p53 clan, mostly in the nucleoplasm, responds to a large and still growing number of alterations in cell homeostasis commonly referred to as stress.[2-5] In any event is the result of such activation—manifested by specific cleavage processing (Notch) and chemical modifications and oligomerization (the p53 clan)—the

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contact with regulatory DNA. This contact is indirect through association with a DNA binding transcription factor in the case of Notch and direct in the case of the p53 family. Finally, in both pathways the cell type and context dependent recruitment of nuclear coregulators entails the stimulation or repression of a very large number, perhaps hundreds, of genes.[6-8] Several of these code themselves for transcriptional regulators, adding a further level of complexity to the networks. It is obvious that transcription factor pathways may crosstalk, for instance, through the sharing of target genes or coregulators and through the engagement in interdependent regulatory loops. Indeed, all of these mechanisms, plus several others, seem to have been realized in the crosstalk of Notch with the p53 clan.

Conspicuously, both groups of pathways are involved—although at first sight in an antagonistic manner—in the regulation of stem cell maintenance, cell differentiation and cell homeostasis that are at the heart of organismal as well as cancer development. While Notch primarily controls the preservation of stemness and the asymmetric development of cell lineages with divergent fates,[1] some members and isoforms of the p53 clan and in particular p53 itself, by contrast reduce the stem cell potential and stimulate differentiation.[9] This antagonism, however, is not inscribed in stone. In dependence, for example, of coregulator recruitment, Notch signalling may counteract proliferation and support rather than prevent differentiation.[10] On the side of the p53 clan, for instance, all three members can be expressed as truncated isoforms capable of counteracting their siblings' transactivating effects.[5] Altogether, the presently available data clearly point to the crosstalk of Notch and the p53 clan being intricately entangled in cell lineage decisions as well as in tumourigenesis. The present chapter intends to provide an overview on this interesting signalling network. But before the crosstalk is discussed, the pathways should be outlined individually.

NOTCH

Notch 1 to 4 are highly homologous single pass transmembrane receptors on the surface of signal receiving cells that can become activated upon contact with one of five canonical Notch ligands (Jagged 1 and 2; Delta-like 1, Dll 3 and 4 in mammals), or one of several noncanonical ligands, on the surface of neighbouring signal emitting cells.[1,11,12] Ligand/receptor interactions within the same cell inhibit rather than induce Notch signalling;[13,14] both the activating and repressing interactions have physiological relevance.[14] Importantly, expression of ligand/receptor is necessary but insufficient for productive signalling, as is evidenced for instance by an often only very sparse, spacio-temporally limited, signalling activity despite of ubiquitous ligand/receptor expression in some tissues.[1] This and the fact that Notch signalling can elicit many different responses already points to a complex regulation of this pathway and a need for integration of many additional signals that goes way beyond a simple canonical ligand/receptor triggering of gene expression. It is now appreciated that Notch signalling involves the coordination of several posttranslational modifications including, but not limited to, activating endocytosis-inducing ubiquitination of the ligand,[15,16] activating glycosylation and chaperone contact of the receptor,[17,18] activating proteolytic cleavages of the receptor and inhibiting ubiquitinations of the receptor such as those by the E3 ubiquitin ligase Numb.[19] Activating ubiquitinases affecting the ligand and inhibiting ubiquitinases affecting the receptor can cosegregate in asymmetric cell divisions and thereby support the formation of disparate daughter cells.[19,20] Altogether, chemical modifications to the Notch pathway control such critical

parameters as the intracellular trafficking, appropriate positioning, surface density and interaction duration of the receptor and ligand, as well as the stability and turnover of pathway-relevant proteins.[21,22] In addition, Notch target gene regulation is controlled by microRNAs and RNA turnover.[23]

Despite the many different complex modifications and impacts necessary to elicit specific responses, core Notch signal transmission per se is remarkably straightforward. Prolonged contact between the epidermal growth factor (EGF) repeats of the ligand's extracellular domain (ECD) on the signal-sending cell with the EGF repeats 11 and 12 of the Notch receptor's ECD on the signal-receiving cell leads to the activation of metalloproteases ADAM10 or 17 (TACE). These cut off the ECD of Notch by S2 cleavage and thereby generate the substrate for the proteolytic γ-secretase complex, which then by S3 cleavage releases the Notch intracellular domain (NICD) after receptor endocytosis.[24,25] This C-terminal Notch fragment is transported into the cell nucleus to act as a coactivator of transcription. NICD cannot contact DNA directly; its transcriptional regulatory function is therefore dependent upon the association with a DNA binding transcription factor. All gene regulatory activities of NICD that have been studied in detail rely on the binding of NICD's RAM23 and ankyrin domains to the beta-trefoil domain of transcription factor RBP-Jκ (CSL); however, other DNA binding transcriptional regulators such as YY1 can also associate with NICD.[26] Transcriptional activation after receptor processing is basically induced by the derepression through NICD of RBP-Jκ prebound to CGTGGGA recognition motifs,[27] involving the recruitment of the coactivator Mastermind-like (MAML) and the histone acetyltransferases p300[28,29] or PCAF.[29,30] After the job is done, the kinase CDK8 phosphorylates NICD to turn it into a substrate for ubiquitin ligase SEL10 that marks it for degradation.[31,32] RBP-Jκ may then stay DNA associated and act again as a transcriptional repressor on which a corepressor complex that may include histone deacetylases, the H3K4 demethylase LSD-1 and H3K27 polycomb methylases—is assembled.[33-35] The most intensely studied among the many Notch target genes are *Hes 1* and *5* and *Hey 1*, themselves encoding HLH-type transcriptional repressors.[36,37] These repress, for instance, proneural genes in progenitor cells of the nervous system in response to active Notch.[1,38] Inhibitory Notch signalling typically is induced by the Delta ligands whereas positive signalling is induced by the Jagged ligands.[1] Notably and important for the discussion below, RBP-Jκ/NICD complexes can act in concert with other transcription factors (for example, ref. 39). In sum, Notch signalling is an evolutionary highly conserved pathway that keeps equipotent cells from all having the same fate.[11] In dependence of cell type and history, Notch signalling can stimulate or inhibit proliferation, regulate cell survival, sense and respond to molecular asymmetries in daughter cells and determine their fate and regulate differentiation as well as stem cell maintenance in the embryo and in adult self-renewing organs—among probably other functions.

THE p53 CLAN

p53, p63 and p73 are transcription factors that bind as tetramers to very closely related DNA motifs: two consecutive 10-mers (half-sites), preferentially spaced by no more than zero to 2 base pairs, with the consensus r,r,r,C,A/T,T,G,y,c,y in the case of p53; r,r,r,C,G,T,G,y,y,y; t/a,a/t,a,C,A/T,T,G,t,t/a,t; or r,r,r,C,A/G,T/A,G,y,y,y in the case of p63; and a/c/g,g/a,g,C,A,T,G,c/t,c,c/t in the case of p73 (r = purines; y = pyrimidines).[6,40,41] The high degree of homology among the consensus sequences and the degeneracy of

individual binding sites make it no surprise that the family members share a large number of target genes.[40] However, as with Notch, the actual control of a specific gene underlies the regulation by posttranslational modifications and protein/protein interactions that are specific for each transcription factor paralog. In addition, DNA binding is affected by the number of the half-sites, their orientation, their position relative to the target gene and their possible overlap with binding sites for other transcription factors. Spacing between the 10-mers may affect protein conformation and the recruitment of coactivator or corepressor complexes.[6] Epigenetic CpG methylation does not seem to inhibit the binding to DNA strongly.[40]

Like the Notch proteins, the p53 clan proteins display a modular organization. Typically, a p53 relative carries an N-terminal transactivation domain (TD), a central DNA binding domain (DBD) that with approximately 60% is the most highly conserved region among the paralogs and C-terminal regulatory and protein/protein interaction domains.[5] Transcription initiation from internal promoters, alternative splicing and alternative translation initiation sites give rise to a large variety of isoforms which, however, maintain the DBD in most cases. More than ten different isoforms of p53 have so far been identified; six of p63 and at least 29 of p73.[5,42] Only few of the biological functions of the minor isoforms have been cleared up. Again as with Notch, a whole arsenal of—partially interdependent and sequential—posttranslational modifications has evolved which, dependent upon the presence or absence of specific protein domains, may or may not distinctly affect individual p53 clan isoforms. Chemical modifications identified so far and regulating: the proteins' abundance, DNA binding, level of activity as transcription factor, crosstalk with other proteins and subcellular localization, include: phosphorylations, acetylations, ubiquitinations, sumoylations, neddylations, methylations, glycosylations and oxidation/reduction.[43,44] Their effects are best studied in p53, revealing an enormous degree of complexity.[3] For example, in the case of phosphorylations, sequentially build-up poly-phosphorylation patterns at different sites (accompanied, perhaps, by other chemical changes such as acetylations), rather than single marks, establish a code that can regulate p53 function in a tissue specific manner.[45-47] Active p53 regulates genes whose products serve functions in the transcription/translation of other genes; in the cell cycle, cell survival and autophagy regulation; in the control of respiration, anti-oxidation, glucose metabolism and cell adhesion/motility; in the cytoskeleton and endo/exosome compartments; and in the control of angiogenesis.[6]

Another example highlighting the complexity of the p53 clan regulation is the partnership between p53 and its most important negative regulators, the E3 ubiquitin ligases MDM2 and MDM4. Numerous of the many known p53 activating events act pro-p53 and anti-MDM2/4, with a large number of protein/protein interactions involved and with many modifications set on both p53 and the MDM proteins—most prominently phosphorylations and acetylations. The latter, for instance, inhibit the ubiquitination of p53 by MDM2 on at least three levels: through the inhibition of MDM2 itself, through the competitive modification of the C-terminal six ubiquitin targeted lysines on p53 and through the inhibition of p53/MDM2 binding.[48-50] MDM4, although not acting as a ubiquitin ligase to p53, can inhibit p53's transcriptional activity and modulate the p53/MDM2 interaction.[44] Since p53 can transactivate the *MDM2* gene, a negative feedback loop is formed.[43,44] P63 and p73 can also stimulate *MDM2* gene transcription; however, in contrast to p53 and although both p63 and p73 are bound by MDM2, they are not ubiquitin-marked by it for degradation.[5]

Broadly, cell context determines the respective function of the p53 clan members. In the absence of extra stress, i.e., under physiological background stress caused, for instance, by reactive oxygen species (ROS) as a byproduct of respiration, they primarily influence cell fate, differentiation and development. These functions seem to be mainly, though not exclusively, mediated by the DNA binding compentent yet transactivation impaired delta-N isoforms of the proteins (ΔNp63, ΔNp73). In cells or tissue that have been additionally stressed, as for example by overt ROS production, radiation, hypoxia, hypo/hyperthermia, metabolite shortages and imbalances, oncogene dysregulation and virus/bacterial/parasite infections, the p53 clan members and in particular p53 itself, mainly control repair, proliferative capacity and survival. These functions are exerted mostly by the transactivation proficient isoforms (p53, TAp63, TAp73). Many of the damaging stresses are known to initiate and/or support cell transformation and the earliest discovered and one of the most impressive functions of p53 is that of a tumour suppressor which can restrain damaged cells from transformation by the induction of cell cycle arrest, senescence, apoptosis and terminal differentiation.[2,3,51] Lack of proper p53 function leads to tumours in many animals including humans; tumour inducing viruses encode proteins that target p53; and there is quite possibly not a single tumour type existing in which the p53 pathway itself as well as all ascending/descending pathways are fully intact. However, it has been suspected for a long time that p53 might exert functions in addition to tumour suppression and indeed, recent discoveries have shown that it can, for instance, modulate stem cells, contribute to the general robustness and life expectancy of humans (independently of tumour suppression), modulate mitochondrial respiration and glucose metabolism and regulate human fertility.[2,52-54]

P63 and p73 are involved in tumour suppression as well, although more subtly than their cousin.[5] For example, while p53 as a classical tumour suppressor is frequently mutated in human cancers, p63 and p73 are not. Rather, p63 is often overproduced in tumours, which at a first glance may seem at variance with the notion of p63 being a tumour suppressor. However, in the majority of these cancers it appears to be the p63 isoforms capable of DNA binding but incapacitated for transactivation due to lack of the transactivation domain (ΔNp63), that are predominating.[55] These are thought to act in a dominant negative fashion towards transactivation competent isoforms of the p53 clan and to thereby function as oncoproteins. In contrast and like p53, transactivation competent p63 (TAp63) can sensitize cells to apoptosis in response to DNA damaging stress.[56] Moreover, in dependence of genetic background p63+/– mice are tumour-prone, with the tumours often showing loss of heterozygosity for the remaining wild-type allele.[57] Loss of the transactivating isoforms of p63 or overproduction of the dominant negative isoforms was also observed, for instance, in human carcinomas of the bladder.[58] Like p53 but more so than p63, p73 is a DNA damage activated transcription factor that in response to damaging stress can stimulate many of the classical p53 target genes and also the gene for p53 itself.[5,59] Mice with a specific knockout of TAp73 display genomic instability and an increased tumour incidence.[60] Of further note, p63 and p73 seem to have p53 independent roles in DNA repair.[61,62]

In contrast to p53 however, p63 and p73 are indispensable for embryonic development in all organisms studied so far.[63] Lack of p53 prevents mesoderm/endoderm fate determination in the frog *Xenopus*[64] but fails to produce obvious early phenotypes in mice or humans.[65,66] At the level of tissues however, p53 deficiency entails over-proliferation of stem cells, in accord with p53 acting as an inducer of stem cell differentiation.[67,68] Another more subtle function of p53 is in mitochondrial respiration; lack of p53 here

results in the reduced endurance of mice during exercise.[69] Contrary to p53 deficiency, overactivity of p53 does indeed have immediate and dramatic consequences in murine development—the apoptotic loss of the early embryo. One of the most remarkable functions of the p53 antagonists MDM2 and MDM4 during embryonic development lies in the prevention of this, in the keeping in check p53 provoked apoptosis already at very early stages of development.[70] At later stages, for example during neuronal development, the DNA binding yet transactivation defective dominant negative isoform of p73, ΔNp73, serves as a p53 (and p63) restraining factor by inhibiting p53 (p63) mediated apoptosis.[71] Both p53 and TAp63 help shape the nervous system by inducing apoptosis; however, TAp63 is self-sufficient in this respect while p53 depends upon TAp63 for this function.[72]

P63 function during development is critical not only for the efficient apoptosis of developing sympathetic neurons[73] but also for epithelial stem cell maintenance,[4,74] squamous epithelial differentiation and skin renewal,[75-77] with ΔNp63 mainly controlling the expansion of epithelial layers and TAp63 somehow pushing differentiation. Above that, TAp63 acts as the guardian of the female germ line by inducing apoptosis in damaged resting oocytes.[78] P73 deficiency in mice results in neuronal and olfactory dysfunctions and in chronic infection and inflammation.[5] Altogether, the stem cell/differentiated cell bifurcation is regulated in part by the balance between the ΔNp63/TAp63 antagonists in the skin and—in an analogous manner—by the balance between the ΔNp73/TAp73 antagonists in the developing nervous and immune systems. But cell fate determination is also the domain of the Notch pathway.

CROSSTALK OF NOTCH WITH THE p53 CLAN IN DEVELOPMENT, DIFFERENTIATION AND HOMEOSTASIS

All Notch and p53 clan members act first and foremost as transcription factors and accordingly, much of the highly complex cross-regulation between them seems to happen at this level. Differentiation and homeostasis of the skin is a well studied example for the interconnection of p53, p63 and Notch 1. Neural development may serve as an example for the crosstalk of p53 (TAp63), p73 and Notch. The maintenance of the stem cell compartment is yet another example that highlights a quite different interconnection between p53 and Notch. Overall, while the crosstalk between Notch and the various p63/p73 isoforms is more complex, the picture emerging for Notch and p53 seems to be that in development, aspects of Notch activity are required for both differentiation (in conjunction with increased p53 activity) and for the maintenance of stemness (here in conjunction with reduced p53 activity). Notch activity in conjunction with reduced (or absent) p53 activity is also often observed in cancers, which in this respect thus resemble stem cells (see below).

The skin consists of a basal layer, itself composed of self-renewing keratinocytes with limited proliferative capacity (transient amplifying cells) and stem cells with high proliferative capacity that have to be preserved and of outwardly migrating layers of mostly resting cells at various stages of differentiation. In the skin, the p53/p63/Notch axis plays an important regulatory role in the maintenance of the stem cells as well as in the establishment of the differentiation gradient. Within the dynamic interplay of these transcription factors, Notch signalling is essential for skin differentiation.[79-82] P53 and TAp63 may support this Notch mediated differentiation of keratinocytes by the direct binding to the *Notch 1* gene promoter and the stimulation of Notch 1 expression.[76,83,84]

Notch in turn can, in a feed-forward setting, stimulate p53 transcription via derepression of RBP-Jκ bound to the p53 gene.[85] In parallel, Notch may also increase p53 activity through inhibition of expression of the p53 antagonist MDM2 by the Notch targets Hes1 and Hey1.[86] Later, the activity of Notch may be limited through the downregulation, by p53, of the gene for the γ-secretase that is critical for Notch signalling.[87-89]

In the undifferentiated proliferating basal layer of the skin, the dominant negatively acting, because DNA binding but transactivation impaired, ΔNp63 rules. Most effects exerted by the transactivation competent p53 clan members are inhibited by it.[90,91] In addition, ΔNp63 may inhibit differentiation by the blunting of Notch signalling through binding to various Notch target genes including *Hes1* and *p21Waf1/Cip1*.[76,92,93] TAp63 that is minor to ΔNp63 in this proliferating compartment of the skin, may become more dominant as ΔNp63 levels decrease in the course of differentiation.[94] TAp63 can transactivate the *Jag 1* and *Jag 2* genes encoding the respective Notch ligands and may thereby contribute to Notch signalling in neighbouring cells.[95,96] TAp63 induced Jag 2 expression on thymic epithelial cells is also important for T-cell maturation.[97] TAp63 overexpression, by contrast, inhibits skin differentiation[77]—perhaps reflecting that overproduction of the Jagged ligands can act inhibitory rather than activating on Notch signalling.[13,14]

If ΔNp63 inhibits the Notch signalling required for differentiation, how then is p63 controlled during differentiation? It turns out that Notch 1, contrary to its stimulating effect on the *p53* gene, can inhibit *p63* gene expression altogether,[98] through the downregulation of the interferon pathway transcriptional regulators IRF7 and 3, the upregulation of nuclear factor kappa B (NFκB) and the stimulation of *p63* regulating microRNAs.[98,74,99-101] This negative feedback may help shift the balance from the differentiation inhibitory ΔNp63 to the differentiation supporting Notch and p53. Further feedback loops, which *in toto* cause the oscillation of Notch signalling observed in development,[102,103] help to self-limit these processes. For instance, Notch can directly bind to p53 and inhibit its DNA binding ability.[104] Whether, in turn, p53 can inhibit Notch activity through this binding is not known, but transcriptional activation of Notch target genes relies upon the coactivators MAML and p300 (see above) which are both also associating with p53,[105-107] pointing to factor competition as another possible mechanism of crossregulation.[28] Notch and the p53 clan may furthermore be cross-controlled through the major negative regulator of the p53 family members, MDM2. This ubiquitin ligase could support Notch signalling through the binding and marking for degradation of the negative effector of Notch signalling, NUMB. On the other hand, MDM2 plus NUMB may convert the p53 clan members into negative transcriptional regulators through the generation of a stable trimeric p53/MDM2/NUMB complex.[108-110] In the undifferentiated basal layer of the skin, epidermal growth factor receptor (EGFR) signalling, in addition to ΔNp63, acts as a negative regulator of *p53* and *Notch 1* expression.[111] Simultaneously, EGFR functions as an inhibitor of anoikis, a form of apoptotic death that could happen when the basal layer cells detach from the basal membrane in the course of apical migration. Finally, transcription factor NFκB may have a function in the restraining of p63 and the activation of Notch/p53 during differentiation: it can induce p63 instability and stimulate *Notch ligand* and *p53* expression.[112,113] Figure 1 provides an overview on the interactions between the Notch and p53 pathways in the skin.

In many respects, p73 seems to neuronal development and homeostasis what p63 is to the development and homeostasis of the skin, with the transactivation defective isoform (here: ΔNp73) mostly, though not always, acting as an inhibitor of differentiation, an

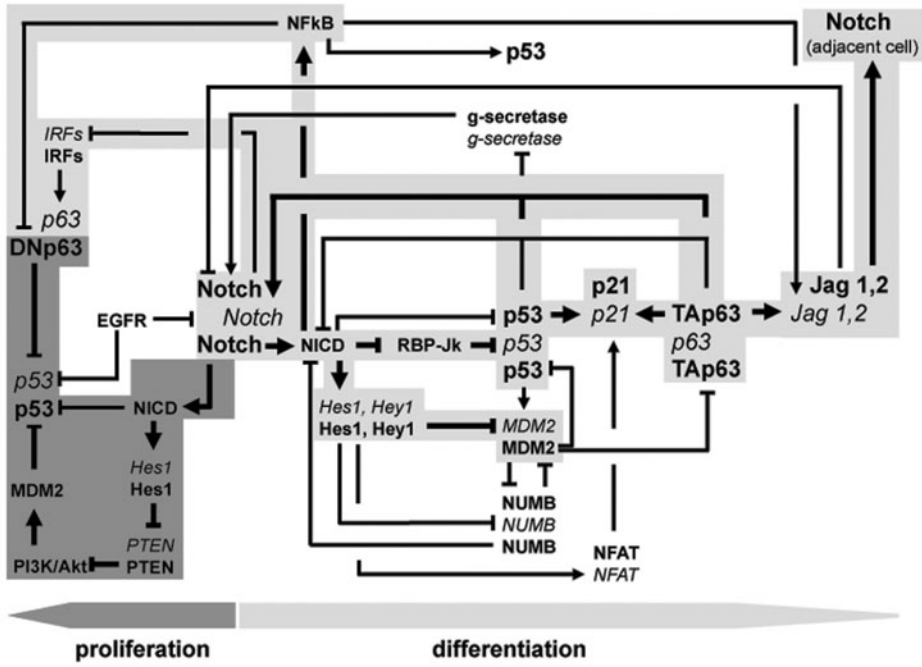


Figure 1. Interactions between the Notch and p53 family pathways in the skin. The major interactions leading to skin differentiation are highlighted in light grey whereas the major interactions that help maintain the proliferative capacity and prevent differentiation are in dark grey. Interactions not highlighted in grey are in part antagonistic to the major interaction pathways and may help limit or temporally restrain the effects produced by these pathways. T bars denote negative and arrows positive interactions. Genes are in italic; proteins are highlighted in black. Notch (NICD), PBP-Jκ, Hes1, Hey1, p53, TAp63, ΔNp63, NFκB, IRFs, NFAT are transcription factors. Notch, EGFR, Jag 1 and 2 are membrane receptors/ligands. MDM2 and NUMB are ubiquitin ligases. PTEN is a phosphatase; PI3K/Akt and γ-secretase are a kinase and a protease, respectively. Further details can be extracted from the main text.

inhibitor of apoptosis induced by TAp63 and p53 and a promoter of proliferation.[71,114] Unlike Notch signalling in the skin however, where it supports differentiation, Notch signalling in the developing CNS inhibits neural differentiation, similar to ΔNp73. The molecular mechanisms underlying this connection are not fully understood; it appears though that TAp73 supports differentiation through the inhibition of Notch signalling. In primary neurons, TAp73 functions as a strong inhibitor of the gene expression induced by the NICD of Notch and thereby as an antagonist to Notch signalling, perhaps due to the direct association of TAp73 and NICD.[115] This inhibition of Notch target genes, but not the binding to NICD, requires the transactivation domain of TAp73. Whether TAp63 and p53, which can also bind and inhibit the NICD, function in an analogous manner in other tissues remains to be seen. Another potential mechanism by which TAp73 inhibits Notch in neurons is through the ligands. Like TAp63 but unlike p53, TAp73 can transactivate the genes for the Notch ligands Jag 1 and Jag 2[96] and overproduction of these can inhibit Notch signalling in the same cell.[13,14] Notch signalling in the CNS not only impacts on differentiation but also on spatial learning, long term potentiation and the formation of memory.[116,117] Moreover, amyloid-β protein associated with Alzheimer's disease activates

p73 through the tyrosine kinase c-Abl.[118] Altogether, it is thus conceivable that the Notch/p73 axis has a role in higher brain functions as well.

Apart from proliferation and differentiation, it is apoptosis that most impressively shapes the nervous system. Programmed cell death is important, for example, for the controlled elimination of a fraction of early neural progenitor cells and this also depends on Notch signalling.[119] Terminally differentiated neurons, by contrast, are insensitive to NICD provoked apoptosis. The induction of apoptosis by Notch in the neural progenitors is relayed through and dependent upon, the activation of p53.[119] In this setting, the NICD leads to the accumulation of nuclear p53. Whether this is mediated by the repression of transcription of the p53 antagonist MDM2, through the NICD-activated Hes1 and Hey1 transcription factors[86] and/or mediated by the direct stimulation of the *p53* gene through the derepression of RBP-Jκ bound to *p53's* promoter,[85] or still other mechanisms, is currently unknown.

While Notch and p53 are protagonists in skin differentiation (where ΔNp63 acts as a major antagonist) and in the apoptosis of neural progenitor cells (where ΔNp73 is a major antagonist), Notch and p53 are themselves antagonists in another interesting example of transcription factor entanglement: the preservation of stem cells. For instance, it is well known that low oxygen pressure, hypoxia, is required to maintain various stem cell and precursor cell populations in the undifferentiated state.[120,121] Under hypoxia, lack of oxygen deprives prolyl hydrolases of their substrate needed to hydroxylate and thereby mark for proteasomal degradation the alpha subunit of the transcription factor hypoxia inducible factor (HIF-1).[122] As a consequence, HIF-1α accumulates and binds to the NICD of Notch; both then associate with DNA bound transcription factor RBP-Jκ and stimulate gene expression above the levels that would be reached with NICD alone.[123] Thus, hypoxia supports the Notch transcriptional program, including the expression of Hes1 and Hey2 and this is critical for the maintenance of the undifferentiated state of stem cells.[123] Hypoxia, however, also constitutes a powerful stress signal for the activation of p53 that can cause the differentiation of stem cells, for example by down-modulating the expression of the stem cell preserving *Nanog*.[9] Furthermore, p53—like the NICD—can bind to the HIF-1α protein, but unlike the NICD then causes its proteasomal degradation.[124] (It is unknown whether the binding of the NICD and p53 to HIF-1α is competitive.) Since HIF-1α acts as a supporter of Notch signalling (see above), this p53 induced breakdown of HIF-1α could weaken a Notch response. In this antagonistic interplay, it may thus be advantageous for Notch to hold p53 in check as, for instance, by the direct association of the NICD with p53.[104] In addition, the hypoxia induced, Notch/Hes1 mediated hyperactivity of the PI3K/Akt kinase pathway observed in stem cells can suppress p53 activity via the activation, through phosphorylation at Ser 166 and Ser 186, of the p53 antagonist MDM2.[125-127] It is no surprise that these (and other) mechanisms of suppression of p53 by Notch are also implemented in cancers.

CROSSTALK OF NOTCH WITH THE p53 CLAN IN CANCER AND OTHER DISEASES

P53 acts as a classical tumour suppressor while Notch can act as a tumour suppressor as well as a tumour promoter. It turns out that much of both functions of Notch are mediated through the p53 pathway—either by activating or by inhibiting it. In addition, Notch can take influence on tumorigenesis through p63 and probably also p73. Conversely, the p53

clan may exert some of its tumour suppressive activities through Notch. This delicate interdependency shall be outlined in the follow.

Among the important transcriptional targets of p53 for tumour suppression is the gene for the inhibitor of cyclin dependent kinases, p21Waf1/Cip1 (CDKN1A hereafter), a powerful blocker of the cell cycle in G1 and G2 phase and thereby a strong anti-proliferative element of the p53 pathway. In keratinocytes, the same gene is also a direct transcriptional target for the RBP-Jκ/NICD complex of the Notch pathway.[81,128,129] In parallel and cooperatively, active Notch suppresses *Calcipressin* expression through Hes1 and thereby causes an increased calcineurin/NFAT activity on the *CDKN1A* promoter.[130] In the end, it turns out that Notch signalling is essential for the suppression of keratinocyte transformation; inhibition of Notch signalling results in the formation of aggressive tumours.[83,128,131,132] In hematopoietic progenitor cells, by contrast, *CDKN1A* transactivation in the presence of activated Notch is not direct but instead through p53, while the induction of differentiation, another important tumour suppressive mechanism by activated Notch, is p53 independent.[133] As discussed above, activation of p53 by Notch can involve NICD mediated derepression of RBP-Jκ bound to the *p53* gene promoter[85] and/or the repression of the gene for the major p53 antagonist, MDM2, by the NICD targets Hes1 and Hey1.[86] In turn, active p53 can transactivate the *Notch 1* gene promoter in several cell types.[83,84,134] The resulting positive feedback may then be modulated, for instance, by p53 degradation through transforming human papilloma virus (HPV) variants, or by p53 induction following UV radiation. As a consequence, in the first case, lack of p53 entails lack of Notch 1 response supports tumorigenesis, whereas in the second, increased Notch 1 response due to increased p53 activity supports anti-tumorigenic differentiation.[84,134,135] Finally, of further importance in this context is the regulation of apoptosis. For example, Notch 1 can act as a survival factor in UV damaged cells by suppressing the proapoptotic forkhead box O 3a (FOXO3a) transcriptional regulator[134] and the combined stimulation of *CDKN1A* by the Notch/p53 collaborators constitutes itself a strong anti-apoptotic signal in proliferating cells.[136]

Another pathway through which both Notch and p53 can act tumour suppressive is the Rho GTPase pathway. Notch and p53 can suppress cell invasiveness via the inhibition of Rho signalling. In the case of Notch this seems to involve primarily the Hes1 induced transcriptional inhibition of the Rho activating kinases ROCK1/2 and MRCKα and since p53 is able to transactivate *Notch*, these two kinases are also under negative control by p53.[83,137] Tumour suppression by Notch could also involve the downregulation of the transcription of the Δ*Np63* isoform whose product acts to the tumour suppressing p53 and p73 in a dominant negative manner.[98,138] This results from the suppression by Notch of transcription factors of the interferon response cascade and perhaps the activation of NFκB. In turn, ΔNp63 may suppress Notch1 expression through precisely this dominant negative activity, by inhibiting the transactivation of *Notch1* by p53. By contrast, TAp63 may act as tumour suppressor in concert with Notch for instance by stimulating the *CDKN1A* gene.[5] Whether TAp73, itself a tumour suppressing p53 clan member[60] and stimulator of *CDKN1A* transcription,[5] can cooperate with Notch is unknown.

A further level of coregulation of Notch and the p53 clan that is of potential relevance for tumour suppression is the activating/repressing modification of MAML, the coactivator of transcription employed by both Notch and p53 and perhaps also p63 and p73.[106] Finally, tumour suppression by the transforming growth factor beta/Smad (TGF-β/Smad) pathway involves Notch as well as p53. For example, full transcriptional

activation of the *CDKN1A* gene in response to TGF-β signalling requires synergistic action on the *CDKN1A* promoter of Smad, Notch and p53.[139,140]

The importance of Notch and p53 in tumour suppression is also highlighted by their combined inactivation in certain transformation processes. In keratinocytes, aberrant constitutive signalling by the epidermal growth factor receptor (EGFR) pathway leads to the downregulation of both Notch and p53 while conversely, suppression of EGFR signalling can cause Notch/p53 mediated growth suppression or, under additional selective inhibition of Notch, p53 dependent apoptosis.[111,141-143] Downregulation of p53 alone—with Notch signalling remaining intact—is typically observed in cancer cells in which Notch signalling acts as a supporter of transformation rather than a tumour suppressor.

While in tumour suppression, Notch may activate p53 through the transcriptional suppression of p53 antagonist *MDM2* by the Notch targets Hes1 and Hey1,[86] in tumour promotion by contrast, Notch may suppress p53 by inducing nuclear translocation of cytoplasmic MDM2 protein through the activation of the PI3K/Akt kinase pathway. This is achieved by the inhibition of the expression of the PTEN phosphatase by the Notch target Hes1 that normally restrains this pathway.[127,144] Concomitantly, p53 suppression may be achieved by the inhibition of the gene for a key negative regulator of MDM2, ARF, by Notch[145] and not least, active Notch can inhibit p53 directly by binding to it and inhibiting its phosphorylation and nuclear localization.[104] Clearly, these antagonistic activities of Notch in tumour suppression and tumour support are context dependent and must involve inputs from other pathways.

There is no doubt that Notch in many cancers functions as a powerful oncoprotein. In many settings, it does so by inhibiting p53 and in particular the proapoptotic activity of p53. A prominent example is T-cell acute lymphoblastic leukemias (T-ALLs), in most of which Notch 1 is ligand independently overactive due to mutation or mutational inactivation of Fbw7, a Notch degrading ubiquitin ligase.[146] As many cell types of the hematopoietic lineage, lymphoblasts are much more sensitive to p53 induced apoptosis than to p53 mediated cell cycle arrest. To prevent apoptosis, active Notch 1 decreases p53 activity and protein levels through the PTEN/PI3K/Akt and possibly ARF pathways mentioned in the previous paragraph. Lack of Notch 1 overactivity in T-ALL is therefore often compensated, for instance, by mutation of *PTEN*.[127] Notch 1 activity normally is blocked in the lymphoid lineage by the repressive transcription factor and lymphoid specific tumour suppressor Ikaros binding competitively to the same sequence motif as RBP-Jκ/NICD.[147] This suggests that Ikaros can act tumour suppressive in this tissue in part by preventing p53 from being down-modulated by Notch. However, the function of Ikaros is lost in almost all T-ALL,[148-150] thus allowing Notch 1 to exert its p53 suppressive activity.

The effects of Notch against p53 provoked apoptosis are also relevant in other cancers including breast carcinomas and gliomas. In these tumour entities, Notch activation and p53 loss are often mutually exclusive, indicating lack of selection pressure against one event in case of the presence of the other.[151] Furthermore, the general inhibitory effect of MDM2 on the transactivation of genes by the p53 clan members TAp63 and TAp73 suggests that Notch might be able to affect these as well as p53 itself. Approximately half of the T-ALL have the potentially tumour suppressive *p73* gene inactivated due to hypermethylation.[152] Whether MDM2 activation in response to Notch has a role in the remainder has not been investigated.

Constitutively active Notch 4 or, less frequently, Notch 1 is found in a large proportion of breast cancers, often in conjunction with the proproliferative c-Myc, CycD1 and active H-Ras oncoproteins.[153-155] Since oncoproteins are known to be able to activate p53

by activating ARF and thereby inhibiting MDM2 and Notch can do the opposite (see above), Notch signalling may protect the transforming, oncoprotein producing cell from p53 mediated cell cycle arrest and apoptosis. Expression of Notch 1 and its ligand Jag 1 are correlated with poor survival of breast cancer patients.[156] Another interesting player in transformation is the ubiquitin ligase Numb. It normally acts as a negative regulator of Notch signalling; in turn, the cellular levels of Numb are under negative control of Notch.[157] Therefore, Numb expression is frequently lost in the portion of breast tumours with constitutively overactive Notch.[158] However, Numb also affects p53.[110] It can bind to the MDM2/p53 complex and stabilize p53, plus apparently keep p53 active as a transcription factor, by inhibiting MDM2.[109] Loss of Numb expression thus is advantageous for a cancer cell in at least two ways: through maintaining Notch signalling and inhibiting tumour suppressive p53. Numb can also alter the function of the ubiquitin ligase Itch that controls and marks for degradation, the p73 tumour suppressor.[159] Altogether, Notch signalling can support several types of cancers including colorectal and pancreatic cancers, melanomas and different lymphomas and leukemias.[158] One common denominator of Notch function in all these malignancies seems to be the inhibition of p53 which might otherwise become activated due to the aberrant expression of various oncoproteins.[160]

The Notch/p53 clan entanglement has a profound influence on other pathological cellular malfunctions apart from cancer. For instance, as briefly discussed above, Notch signalling is important in the adult CNS[116,117] and there may have an impact on p73 that can become activated by the amyloid-β protein accumulation associated with Alzheimer's disease.[118] Diabetic nephropathy may serve as another example. Here, glomerular disease is caused by the extensive apoptosis of podocytes, which was shown to be induced by activated Notch and mediated through the activation of p53.[161] Full length Notch (not the NICD) may lead to a reduction of T-cell apoptosis in the thymus and to autoimmune disease, partly perhaps by the suppression of p53 through the PI3K/Akt pathway.[162] Aberrant Notch signalling in combination with active p53, has also been implicated in paediatric as well as adult cardiac diseases.[163] The common theme of these diseases seems to be a disturbed tissue homeostasis caused by overt apoptosis. Other diseases with a potential contribution of Notch may, in contrast, be attributed to lack of proper differentiation of a tissue rather than apoptotic cell loss. A group of related inherited syndromes in humans is caused by germ line mutations in the *p63* gene and shows limb malformations, ectodermal dysplasia and facial clefting.[6] These may involve at least in part the Notch pathway as p63 functions as a selective modulator of Notch and as a direct transcriptional activator of the Notch ligands Jag 1 and 2. It seems likely that in the near future many other noncancer diseases will be identified to involve malfunction of the Notch/p53 clan pathways.

CONCLUSION

Notch signalling plays an important role in the communication between cells that ultimately decides about the fate of individual cells and cell types within a developing or aging tissue. The members of the p53 family of transcription factors receive signals from Notch on several levels and either mediate or contribute to the mediation of many of its effects in stem cell maintenance, differentiation and the regulation of tissue homeostasis. Conversely, the p53 family also talks to Notch on several levels. That way, a highly complex and dynamic regulatory network of feedforward and feedback interactions is

implemented. Typically, the coordinated transactivation of genes by Notch and p53, p63 or p73 is associated with the initiation of cellular programmes that lead to the inhibition of proliferation and to differentiation. In contrast, gene transactivation by Notch alone is often observed in cells in which proliferative capacity is to be maintained and differentiation is unwanted, including cancer cells. The reasons for p53 and its transcriptionally competent cousins not being active in this last setting can be manifold. They include their direct and/or indirect inhibition by Notch itself, their loss of function altogether, for instance through the expression of dominant negative isoforms of the p53 family and probably regularly a combination of both. Current strategies for the therapeutic intervention in cancers and other diseases involving these pathways tend to focus on either Notch or p53. A deeper understanding of the Notch/p53 network will in the future likely provide rationales for sensibly combining existing therapies and designing new ones.

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CHAPTER 16

LIGAND-DEPENDENT NOTCH SIGNALING IN VASCULAR FORMATION

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Abstract: The Notch signaling pathway is a critical component of vascular formation and morphogenesis in both development and disease. Compelling evidence indicates that Notch signaling is required for the induction of arterial-cell fate during development and for the selection of endothelial tip and stalk cells during sprouting angiogenesis. In mammals, two of the four Notch receptors (Notch1 and Notch4) and three of the five Notch ligands (Jagged1, Dll1 and Dll4) are predominantly expressed in vascular endothelial cells and are important for many aspects of vascular biology. During arterial cell-fate selection and angiogenesis, the roles of Notch1 and Notch4 are thought to be similar and the role of Dll4 is well-characterized. However, the molecular mechanisms that determine the functional similarities and differences of Notch ligands in vascular endothelial cells remain largely unknown; consequently, additional research is needed to elucidate the ligand-specific functions and mechanisms associated with Notch activation in the vascular endothelium. Results from recent studies indicate that Dll1 and Dll4 have distinct roles in the specification and maintenance of arterial cell identity, while Dll4 and Jagged1 have opposing effects on tip- and stalk-cell selection during sprouting angiogenesis. This chapter will focus on the newly discovered, distinct roles of several Notch ligands in the regulation of blood vessel formation and will provide perspectives for future research in the field.

INTRODUCTION

Notch signaling is evolutionarily conserved and critical for cell-fate determination, differentiation and many other biological processes.[1] The mammalian Notch signaling

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pathway is composed of four Notch receptors (Notch1-4) and five ligands (Jagged1 and 2 and Delta-like [Dll] 1, 3 and 4). All of the ligands are transmembrane-type proteins and, consequently, Notch signaling is often mediated by cell-cell interactions. Transmission generally occurs between neighboring cells that express high levels of either the receptor or the ligand, although receptor-ligand coexpression occurs in some cells, such as vascular endothelial cells. Over the last decade, numerous studies have demonstrated that Notch signaling is critically involved in vascular development and disease.[2-6] For example, Notch signaling is required for arterial cell-fate determination during embryonic development and the Notch pathway controls both developmental and pathological angiogenesis by modulating the selection of endothelial tip and stalk cells in newly sprouting blood vessels. Regulation of the Notch pathway in blood vessels has been well characterized; however, the specific roles of each Notch ligand during vascular formation and morphogenesis are unknown. Recent studies provide insight into the distinct functions of Notch ligands in blood vessels and this chapter summarizes the current understanding of how several ligands differentially activate Notch signaling in the vasculature.

BASIC MECHANISMS OF THE NOTCH RECEPTOR LIGAND-SIGNALING PATHWAY

Notch signaling is initiated by interactions between a Notch ligand expressed on the surface of one cell (the signaling cell) and a Notch receptor expressed on the surface of a neighboring cell (the receiving cell). Upon ligand binding, Notch is sequentially cleaved and the Notch intracellular domain (NICD) is released into the cytoplasm. The NICD enters the nucleus, where it interacts with the transcription factor CSL (named after mammalian CBF1, *Drosophila* Su(H) and *Caenorhabditis elegans* LAG1) to form a transcriptional activation complex that induces expression of the bHLH transcription factors (Hes and Hey families). This signaling mechanism is considered the "canonical" Notch pathway; noncanonical Notch signaling has also been reported.[7]

The extracellular domains of mammalian Notch ligands have several distinct features that participate in receptor binding (Fig. 1). Their N-terminal regions contain a conserved module and a second conserved module, the DSL (Delta/Serrate/LAG-2) domain, is located adjacent to the N-terminal region. Both Notch ligands and receptors contain multiple EGF-like repeats and the ligands Jagged1, Jagged2 and Dll1 have tandem EGF repeats that form the DOS (Delta and OSM-11-like proteins) domain.[8] Jagged1 and Jagged2 also contain a cysteine-rich domain located between the EGF-like repeats and the transmembrane domain. Both the DSL and DOS domains are critical for receptor binding,[9] and the structural diversity of Notch ligands is determined by the presence or absence of the cysteine-rich DOS domains.

Activation of Notch signaling through cell–cell interactions (*trans*-interactions) has been well characterized; however, Notch ligands also regulate the Notch pathway by binding to Notch receptors within the same cell (*cis*-interactions).[10,11] In general, *trans*-interactions between Notch ligands and receptors activate Notch signaling, whereas *cis*-interactions are believed to inhibit Notch signaling.[9] The precise mechanisms that mediate Notch activation by the *cis*-interactions remain unclear and further studies need to be performed.[12]

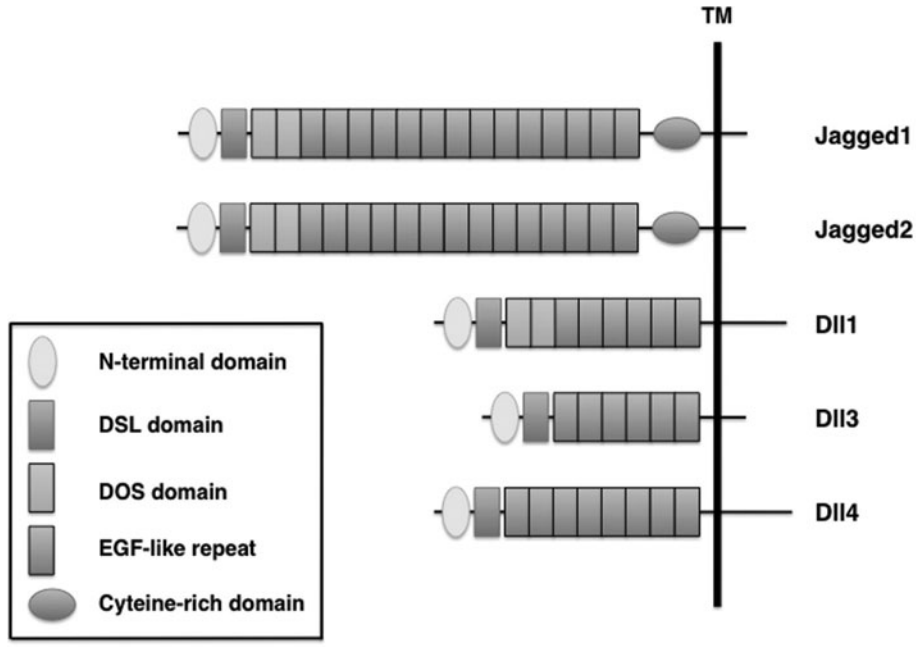


Figure 1. Domain organization of mammalian Notch ligands. Five mammalian ligands are classified into two categories, Delta-like (Dll1, Dll3, Dll4) and Serrate-like (Jagged1, Jagged2), based on structural homology to the two Drosophila ligands, Delta and Serrate. All Notch ligands have an N-terminal domain, a DSL (Delta/Serrate/LAG-2) domain and EGF-like repeats. Jagged1 and Jagged2 contain a cysteine-rich domain, whereas Jagged1, Jagged2 and Dll1 have two DOS (Delta and OSM-11-like proteins) domains located immediately following the DSL domain. Adapted from Kume.[70]

NOTCH RECEPTOR AND LIGAND EXPRESSION IN BLOOD VESSELS

Notch1 is broadly expressed in many tissues, including the heart and vascular endothelial cells, while Notch4 expression is restricted to vascular endothelial cells,[13-15] and Notch3 is predominantly expressed in vascular smooth muscle cells.[16] Transcriptional regulation of *Notch4* in vascular endothelial cells is controlled by fibroblast growth factor 2 (FGF2), the signal-dependent transcription-factor activator protein 1 (AP-1) and the glucocorticoid receptor.[14,15,17] Four of the five known mammalian Notch ligands (Jagged1, Jagged2, Dll1 and Dll4) are expressed in vascular endothelial cells;[13,18-20] Jagged1 is also expressed in smooth muscle cells surrounding the arteries and plays an important role in smooth muscle cell maturation.[21] The molecular mechanisms that control the expression of Notch ligands in vascular endothelial cells and smooth muscle cells have been frequent topics of recent research (Table 1). For example, Dll4 expression during arterial specification and for tip-cell selection during vessel sprouting[22-28] is mediated by vascular endothelial growth factor (VEGF). Importantly and as described below, the expression patterns of the Notch ligands vary both spatially and temporally and, consequently, the localization of each ligand is likely to be critical for Notch activation in blood vessels (Figs. 2-5).

Table 1. Signaling pathways/factors that regulate Notch ligand expression in vascular endothelial and smooth muscle cells

Pathway/Factor	Ligand	Cell Type	Biological Effect	References
VEGF	Dll4 (↑)	Endothelial cells	Arterial specification	22, 24, 27
			Endothelial tip cell formation in sprouting angiogenesis	23, 25, 26, 28
TNFα	Dll4 (↓)	Endothelial cells	Tip cell and stalk cell selection	50
	Jagged1 (↑)			
VEGF + FGF2 (synergistic)	Dll1 (↑)	Endothelial cells	Ischemia-induced postnatal arteriogenesis	38
PDGF/ angiotensin II	Jagged1 (↓)	Smooth muscle cells	Growth regulation	43
Jagged1 (endothelial)	Jagged1 (↑)	Mural cells	Smooth muscle cell maturation	21

↑, Upregulation; ↓, Downregulation.
VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; FGF, Fibroblast growth factor; PDGF, platelet-derived growth factor Adapted from Kume.[70]

NOTCH1, NOTCH4 AND THE LIGANDS Dll1 AND Dll4 DURING ARTERIAL SPECIFICATION AND MAINTENANCE

Results from recent studies in zebrafish suggest that activation of Notch signaling by the Sonic hedgehog (Shh) and VEGF pathways is essential for arterial specification during development.[29,30] Two Notch receptors, Notch1 and Notch4, are predominantly expressed in arterial endothelial cells of early mouse embryos. *Notch1* mutant mice die with cardiovascular defects during early development,[13] and endothelial-specific ablation of *Notch1* in mice leads to embryonic lethality and vascular abnormalities that are associated with angiogenesis.[31] An endothelial-specific *Notch1*$^{+/-}$ mutation has also been associated with impaired postnatal neovascularization in a murine hind-limb ischemia model.[32] These results demonstrate the cell-type specific role of Notch1 in the vascular endothelium during development and postnatal life (Table 2). *Notch4* mutant mice display no conspicuous phenotype, but the vascular defects observed in compound *Notch1; Notch4* mutant embryos are more severe than those in *Notch1* mutants,[13] which suggests that the functions of Notch1 and Notch4 overlap during vascular development. Endothelial expression of a constitutively active Notch4 mutant from the *Flk1* (*VEGFR2*) locus results in embryonic vascular abnormalities such as disorganized vascular networks and dilated blood vessels,[33] and in adult mice, constitutively active Notch4 expression has been associated with arteriovenous malformations (AVMs) (i.e., abnormal connections between arteries and veins) that are accompanied by ectopic expression of the arterial marker ephrinB2 in veins.[34] Furthermore, the impaired arterial and venous differentiation associated with constitutively active Notch4 expression can be reversed by suppressing Notch4 activity in the endothelium.[34] Collectively, these findings suggest that both reduced

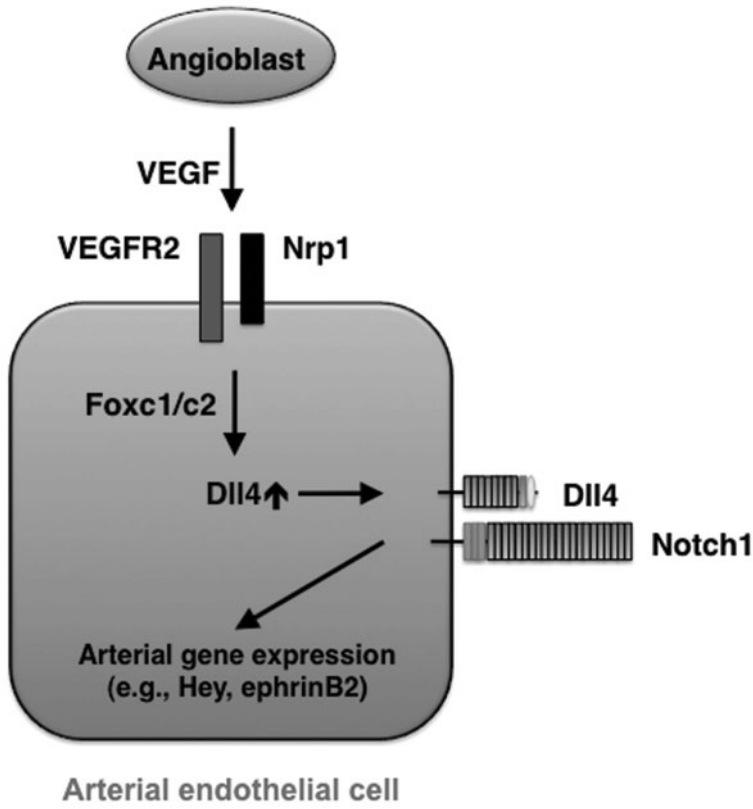


Figure 2. Arterial cell specification mediated by Dll4-Notch signaling. During early development, VEGF (in concert with Foxc1/c2 transcription factors) induces Dll4 expression in endothelial cells and Dll4-Notch signaling promotes arterial gene expression. Adapted from Kume.[70]

and elevated Notch signaling can lead to vascular abnormalities and, consequently, that the maintenance of proper Notch signaling is critical during vascular development.

Of the four Notch ligands (Jagged1, Jagged2, Dll1 and Dll4) that are expressed in arterial endothelial cells, Dll4 alone is expressed in the dorsal aorta of mice at embryonic day 8.5 (E8.5) and its expression is restricted to vascular endothelial cells;[13] thus, Dll4 is believed to be the ligand for Notch1 and Notch4 during early vascular development (Fig. 2). *Dll4* mutant mice display early embryonic lethality with impaired arterial specification and AVMs that appear in a genotype-dependent manner (i.e., the severity increases with the number of mutant alleles).[35-37] These observations further emphasize the importance of maintaining proper Notch activity levels during vascular development. Foxc1 and Foxc2 transcription factors directly activate the *Dll4* promoter in endothelial cells and their induction of *Dll4* expression is enhanced by VEGF, which suggests that Foxc1 and Foxc2 act upstream of Notch signaling during arterial-cell specification.[22,27]

Dll1 expression is detected in arterial endothelial cells at a later stage (E13.5) of mouse development[19] and continues to be restricted to arterial endothelial cells in

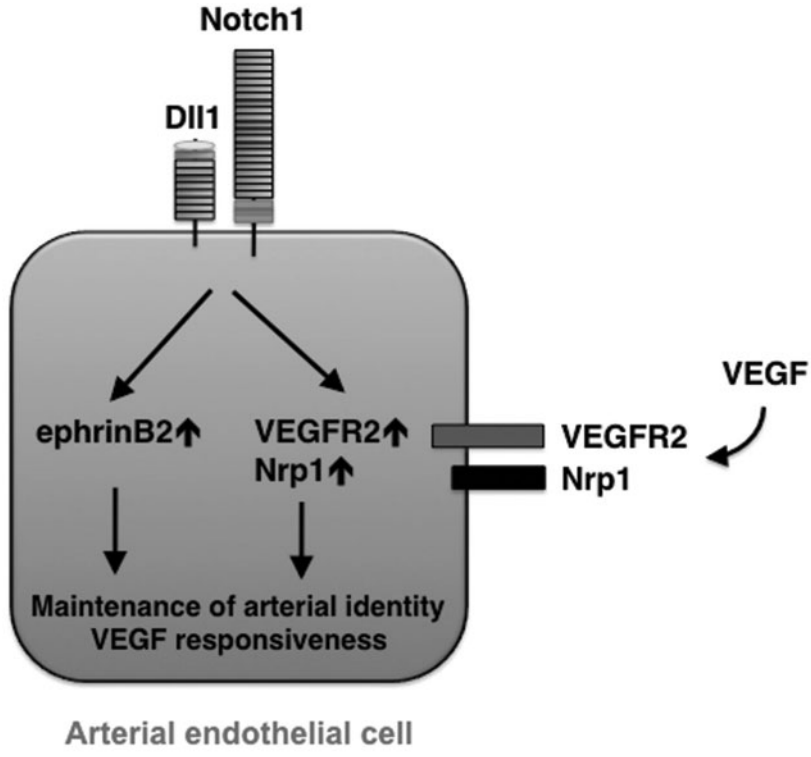


Figure 3. Maintenance of arterial identity mediated by Dll1-Notch signaling. At a later stage of development, Dll1 expression is induced in arterial endothelial cells and is required for maintenance of the arterial phenotype. Dll1 also acts upstream of VEGF by regulating the expression of VEGFR2 and its coreceptor neuropilin 1 (Nrp1). Adapted from Kume.[70]

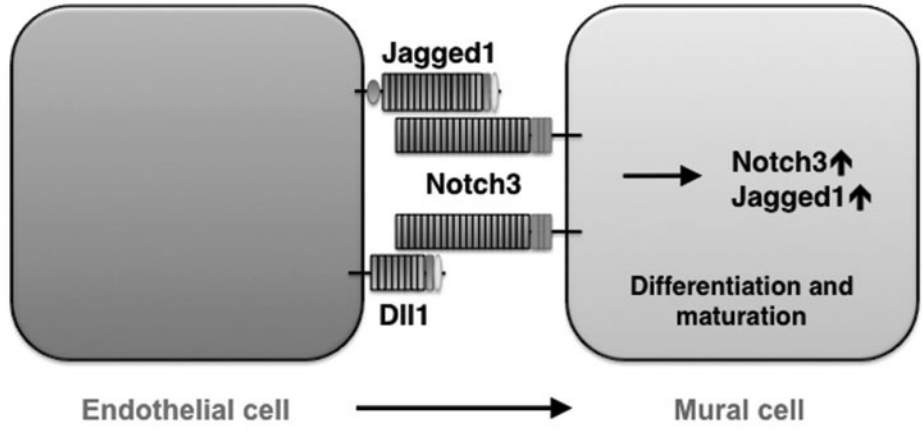


Figure 4. Smooth-muscle maturation mediated by Jagged1/Dll1-Notch3 signaling. Jagged1 and Dll1 in endothelial cells activate Notch3 on mural cells, thereby promoting mural-cell maturation. Adapted from Kume.[70]

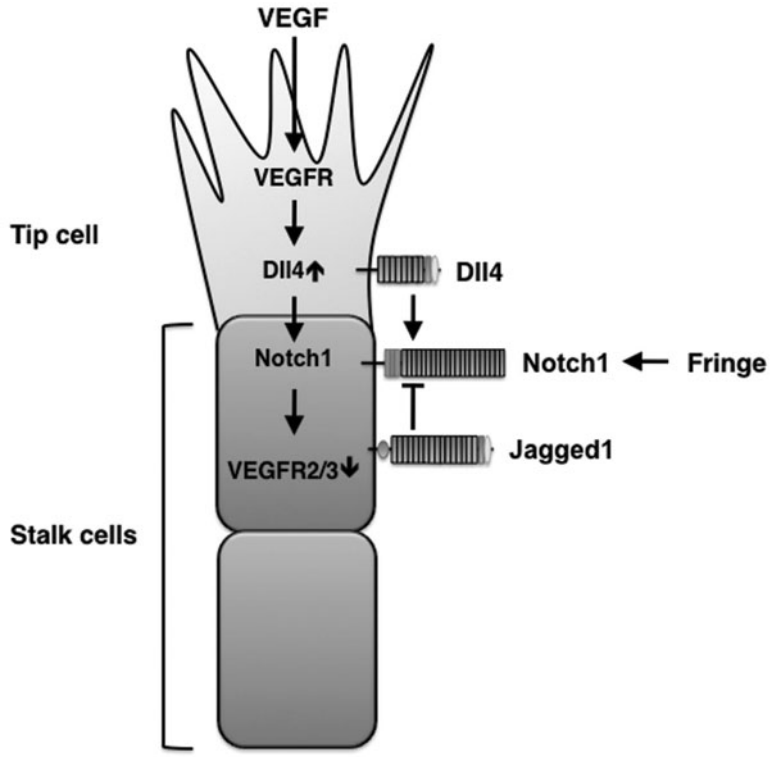


Figure 5. Opposing effects of Dll4 and Jagged1 on sprouting angiogenesis. VEGF signaling induces Dll4 expression in tip cells and Dll4, in turn, activates Notch signaling in stalk cells, which reduces stalk-cell sensitivity to VEGF stimulation and, consequently suppresses the tip-cell phenotype. Conversely, Jagged1 antagonizes Dll4-mediated Notch activation in stalk cells to increase tip cell numbers and enhances vessel sprouting. The antagonistic effects of the two ligands are controlled by Fringe-dependent modulation of Notch signaling. Adapted from Kume.[70]

adults.[38] Dll1 is not critically involved in arterial-cell specification; however, analyses in hypomorphic and endothelial-specific *Dll1* mutant mice indicate that Dll1 is required for the maintenance of arterial identity.[19] Expression of the arterial marker ephrinB2 is reduced and the venous marker COUP-TFII is upregulated, in endothelial-specific *Dll1* mutant mice, despite Dll4 expression in the mutant endothelial cells;[19] thus, Dll4 appears to be essential for initiating the arterial program, whereas Dll1 is required to maintain arterial identity during embryonic development. In addition, Sorensen et al have shown that Dll1-mediated Notch1 activation upregulates VEGF receptor 2 (VEGFR2) and its coreceptor, neuropilin-1, which suggests that Dll1 enhances the responsiveness of arterial endothelial cells to VEGF signaling. Thus, Dll4-mediated Notch signaling occurs downstream of VEGF during arterial specification, whereas Dll1-mediated Notch signaling acts upstream of VEGF to maintain arterial identity (Figs. 2,3). Dll1 is also important for ischemia-induced postnatal arteriogenesis and the induction of ephrinB2.[38]

NOTCH3, JAGGED1 AND Dll1 DURING SMOOTH-MUSCLE DIFFERENTIATION AND MATURATION

Notch3 is predominantly expressed in the vascular smooth muscle of arteries and is not expressed in veins. Mutations in human *NOTCH3* are associated with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a disorder that causes stroke and dementia and is accompanied by the degeneration of vascular smooth muscle cells;[39] adult *Notch3* mutant mice display a defect in the maturation of arterial smooth muscle cells.[40] As noted above, *Jagged1* mutant mice exhibit normal arterial development,[41,42] yet endothelial-specific *Jagged1* mutants have impaired vascular smooth muscle differentiation.[41] This observation indicates that Jagged1 expression in the arterial endothelium activates Notch in neighboring cells and that this function is critical for smooth muscle cell differentiation. Jagged1 expression by endothelial cells induces mural cells (pericytes in the microvasculature or smooth muscle cells in larger vessels) to express Notch3 and Jagged1, which subsequently promotes and maintains the differentiation phenotype of mural cells,[21] whereas platelet-derived growth factor (PDGF) and angiotensin II downregulate Notch3 and Jagged1 expression in vascular smooth muscle cells.[43] Furthermore, a recent study found that expression of the arterial smooth muscle marker smoothelin is impaired in *Dll1* mutant mice,[19] and this decline has also been observed in *Notch3*-mutant arteries.[40] Taken together, these findings suggest that Jagged1 and Dll1 are the primary ligands that regulate Notch3 activity during smooth-muscle differentiation and maturation (Fig. 4).

Dll4 AND JAGGED1 IN TIP- AND STALK-CELL SPECIFICATION DURING SPROUTING ANGIOGENESIS

The formation of new blood vessels, a process known as angiogenesis, involves the sprouting of endothelial cells. In response to VEGF stimulation, filopodia extend from a migratory endothelial cell at the vessel's tip (i.e., the tip cell) and proliferative endothelial cells (i.e., stalk cells) form the trunk of the new vessel. Recent studies in mice and zebrafish clearly demonstrate that Notch signaling interacts with VEGF signaling during tip-cell and stalk-cell specification.[5] VEGF induces Dll4 expression in tip cells, then Dll4 activates the Notch pathway in adjacent endothelial cells to reduce expression of VEGFR2 and VEGFR3, thereby suppressing the tip-cell phenotype, and tip-cell phenotype suppression cell-autonomously promotes the stalk-cell phenotype. Together, these mechanisms balance tip-cell and stalk-cell selection and, consequently, limit the number of sprouting vessels (Fig. 5). Genetic or pharmacological disruption of Dll4-Notch signaling leads to excessive tip-cell formation and vessel sprouting in cultured cells, in zebrafish and mouse embryos and during tumor angiogenesis.[23,25,44-49]

By using endothelial-specific *Jagged1* mutant mice and mice that overexpress *Jagged1* in vascular endothelial cells, Benedito et al demonstrated that Jagged1 enhances angiogenesis and antagonizes the effects of Dll4-mediated Notch signaling during sprouting angiogenesis.[50] Jagged1 is strongly expressed in stalk cells, whereas Dll4 is predominantly detected in tip cells,[50] and the antagonistic interaction between Dll4 and Jagged1 in endothelial cells is mediated by the glycosyltransferase Fringe, which regulates the posttranslational modifications of Notch receptors in a ligand-dependent manner. Fringe enhances Notch activation in response to Delta-like ligands and reduces

Notch activity in response to Jagged ligands;[12] consequently, Fringe increases Dll4-induced endothelial Notch signaling and reduces Notch signaling in response to Jagged1.[50] Jagged1 also appears to promote vascular sprouting by regulating VEGFR3 expression in tip cells.[50] Taken together, these results illustrate the opposing effects of Dll4 and Jagged1 on sprouting angiogenesis.

NOTCH LIGANDS IN PATHOLOGICAL ANGIOGENESIS

Dll4 is expressed in tumor vasculature,[26,36,51,52] and as observed in studies of developmental angiogenesis, the blockade of Dll4-mediated Notch signaling (via systemic administration of Dll4-neutralizing antibodies[45,46] and systemic or local administration of modified Dll4 proteins[45,53] increased tumor-vessel sprouting, which indicates that Dll4-Notch signaling is critical for tip- and stalk-cell selection during tumor angiogenesis. Remarkably, the inhibition of Dll4-Notch signaling increased neovascularization but impaired tumor growth, because the nonproductive angiogenesis reduced tumor perfusion. Conversely, Dll4 activation of endothelial Notch signaling reduces tumor angiogenesis, but increases vessel diameter and perfusion, which enhances tumor growth.[45,54] For these reasons, Dll4 is now recognized as a potential therapeutic target for tumor angiogenesis.[55]

As described above, Jagged1 antagonizes Dll4 during sprouting angiogenesis,[50] and overexpression of Jagged1 in tumor cells has been shown to enhance neovascularization and tumor growth;[56] however, the role of Jagged1 in pathological angiogenesis (including tumor angiogenesis) is not yet fully understood. Current findings suggest that angiogenic sprouting in the tumor is tightly controlled by positive and negative regulation of Jagged1 and Dll4 in both endothelial and non-endothelial cells. Recent studies have shown that a soluble form of Notch1 (Notch decoy) acts as an antagonist of ligand-dependent Notch signaling by (potentially) interfering with Dll1, Dll4 and Jagged1.[57,58] Importantly, the Notch decoy reduces tumor growth without increasing vessel growth, which suggests that the effects of the Notch decoy differ from those induced by Dll4 blockade. It is therefore likely that the proangiogenic function of Jagged1 in tumor cells and endothelial cells could also influence tumor angiogenesis.

NOTCH SIGNALING IN PERIPHERAL ISCHEMIA

Notch signaling is also required for angiogenesis in peripheral ischemia models (Table 2).[32,38] Blood flow recovery and postnatal neovascularization in response to hind-limb ischemia are impaired in both global and endothelial-specific *Notch1*$^{+/-}$ mice, but not in *Notch4*$^{-/-}$ mice.[32] Dll1 is strongly induced in arterial endothelial cells during ischemia-induced arteriogenesis and *Dll1*$^{+/-}$ mice display reduced collateral-artery growth and impaired blood-flow recovery after hind-limb ischemia.[38] Notch activation and ephrinB2 induction are not observed in the collateral arteries of *Dll1*$^{+/-}$ mice.[38]

Since the identification of endothelial progenitor cells (EPCs) in human peripheral blood in 1997, substantial progress has been made in understanding EPC-mediated postnatal neovascularization,[59,60] although the definition of EPCs is still a matter of debate because of the lack of unique cell surface molecules that specifically recognize EPCs.[59,61] The mobilization of EPCs from bone marrow and recruitment of these cells into the sites of

Table 2. Mammalian Notch receptors and ligands involved in vascular development and disease

Component	Phenotype/Role	References
Notch receptors		
Notch1	Proper vascular development; Postnatal neovascularization; EPC function	13, 31, 32, 64
Notch3	Maturation of vascular smooth muscle cells	40
Notch4	Null mice show normal vascular development; *Notch1; Notch4* mutant mice have severe vascular defects; Gain-of-function experiments show vascular abnormalities in development and postnatal life	13, 33, 34
Notch ligands		
Jagged1	Dispensable for arterial specification; Smooth muscle differentiation and maturation; Proangiogenic regulation; EPC commitment	21, 41, 50, 63
Dll1	Maintenance of arterial identity; Arterial smooth muscle differentiation; Postnatal arteriogenesis	19, 38
Dll4	Arterial specification; Tip cell and stalk cell selection during sprouting angiogenesis; Regulation of tumor angiogenesis	23, 25, 35-37, 44-50, 53, 54

EPC, endothelial progenitor cell.
Adapted from Kume.[70]

ischemia-induced neovascularization are critical for vascular repair and regeneration in patients with peripheral vascular and coronary artery disease. Significantly, recent studies show that Jagged 1 in the bone marrow microenvironment regulates EPC commitment and differentiation.[62,63] Jagged 1 and Dll1 are expressed on stromal niche cells of bone marrow and the reciprocal interaction between Jagged 1, but not Dll1, and EPCs expressing Notch receptors stimulates EPC commitment, differentiation and mobilization, thereby leading to EPC-mediated ischemic neovascularization. Moreover, Ii et al have recently shown that Notch1 regulates EPC function and viability during recovery from arterial injury in hypercholesterolemic mice.[64]

Taken together, investigation of Notch signaling in EPCs is likely to lead to the advancement of cell-based therapies for cardiovascular regeneration and analysis of Notch function in therapeutic angiogenesis may promote a new treatment approach for ischemic cardiovascular disease in the future.

CONCLUSION

Studies performed in the past few years clearly demonstrate that the different Notch ligands have distinct functions in vascular development and disease. This understanding has prompted numerous investigations into the mechanisms by which Notch signaling is essential for multiple aspects of vascular biology. However, given that the effects of Notch pathway activation on endothelial cells are context-dependent,[4] many questions remain to be answered. First, the upstream signaling pathways that control the expression

of Notch ligands in blood vessels remain largely unknown; VEGF induces Dll4 expression in endothelial cells (Table 1), but Jagged1 is absent in tip cells where Dll4 is highly expressed, which suggests that the two ligands are regulated differently. Second, the selective activation of Notch in vascular endothelium remains unclear; for example, Notch signaling is not activated in arteries of *Dll1* mutant mice, despite the presence of Jagged1 and Dll4.[19] Third, the role of noncanonical Notch ligands, such as microfibril-associated glycoprotein (MAGP)-2,[9] is poorly understood. MAGP-2 binds to Jagged1, Jagged2, Dll1 and Notch1,[65,66] and is known to modulate Notch signaling in sprouting angiogenesis,[67,68] but the mechanistic basis for the function of MAGP-2 in ligand-dependent Notch activation has yet to be elucidated. Finally, given that Dll4 and Jagged1 have opposing effects on angiogenesis, experiments that specifically inhibit each ligand with selective neutralizing antibodies[69] may be important not only for understanding how Notch is activated in the vasculature, but also for the development of therapeutic strategies designed to control angiogenesis by targeting Notch signaling.

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CHAPTER 18

NOTCH SIGNALING AND BREAST CANCER

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Abstract: It has been more than two decades since *Notch* has been identified as an oncogene in mouse mammary tumor virus-infected mice. Since this discovery, activated Notch signaling and up-regulation of tumor-promoting Notch target genes have been observed in human breast cancer. In addition, high expression of Notch ligands and receptors has been shown to correlate with poor outcome in this malignancy. Notch affects multiple cellular processes including stem cell maintenance, cell fate specification, differentiation, proliferation, motility and survival. Perturbation of these activities is a hallmark of carcinogenesis and evidence continues to accumulate that aberrant Notch activity influences breast cancer progression through these processes.

INTRODUCTION

Breast cancer is a significant health problem and ranks second among causes of cancer death in North American women.[1] With the development of targeted therapies such as anti-estrogens for estrogen receptor (ER) positive and trastuzumab for Her2 positive disease, mortality in breast cancer has decreased significantly. Despite these improved treatments, a number of challenges persist; particularly the development of therapies that target breast cancer lacking ER and/or Her2 expression. The Notch signaling pathway is required for normal mammary gland development and plays a key role in the maintenance of mammary stem cells and progenitor cell fate. Predictably, deregulation of Notch has been implicated in the development of mammary carcinoma, particularly breast cancers of the triple negative (TN; basal-like and mesenchymal ER-, PR- and Her2-negative) subtype. This chapter presents evidence for abnormal Notch pathway activation in breast cancer and rationale for the development of therapies that target this pathway.

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EVIDENCE FOR ONCOGENIC NOTCH IN CARCINOMA OF THE BREAST

The contribution of aberrant Notch signaling to breast cancer was first noted in the murine mammary gland. Here, the *Notch4* gene (also known as the *Int-3* locus) was identified as a common proviral integration site in mouse mammary tumor virus (MMTV)-induced mammary adenocarcinomas.[2,3] Insertion of the provirus into the *Notch4* locus was found to lead to over-expression of a truncated form of Notch4 which consisted of the transmembrane and intracellular domain and was structurally similar to activated, intracellular Notch4 ($N4^{IC}$). Like Notch4, Notch1 also displays oncogenic properties in the mammary gland. Dievart and colleagues used provirus insertional mutagenesis in transgenic mice in which the MMTV long terminal repeat (LTR) drives expression of the *Her2/Neu* gene (MMTV/*Neu* mice) to identify genes that cooperate with *Her2* in mammary tumorigenesis.[4] MMTV/*Neu* mice spontaneously develop asynchronous mammary tumors with a long latency period.[5] However, infection of these mice with MMTV led to hastened onset of tumors containing *Notch1* gene interruption by MMTV, over-expression of a truncated 3' Notch1 transcript and a constitutively active protein product lacking most of the extracellular domains.

Notch transgenic mice have proven useful in exploring the oncogenic potential of $N4^{IC}$, $N1^{IC}$ and $N3^{IC}$ in the mammary gland. Mice harboring a *N4^{IC}* transgene driven by the MMTV promoter demonstrate a dual phenotype with arrested mammary gland development and poorly differentiated adenocarcinomas by 4 to 6 months of life.[6,7] A similar phenotype is observed in transgenic mice expressing $N4^{IC}$ driven by the whey acidic protein (WAP) promoter. Since WAP activity is restricted to secretory epithelial tissues of the pregnant female, virgin transgenic females display no apparent phenotype. However, growth and differentiation of secretory lobules in pregnant animals is absent and mammary dysplasia and tumorigenesis ensues in 100% of breeding females by 25 weeks of age.[8] MMTV/*N1^{IC}* and MMTV/*N3^{IC}* transgenic animals have also been created and have a phenotype similar to $N4^{IC}$ mice in that they exhibit impaired ductal and lobulo-alveolar mammary gland development followed by later development of mammary gland tumors.[9,10] MMTV/*N1^{IC}* tumors occur during pregnancy/lactation and regress when the mammary gland involutes; in subsequent pregnancies they progress to nonregressing adenocarcinomas. Together, these data suggest that ectopic expression of activated Notch1, 3 or 4 leads to cell fate restriction and promotion of tumorigenesis in the mouse mammary epithelium.

Initial evidence for abnormal Notch activation in human breast cancer came from studies in cell lines. In an effort to examine Notch4 gain-of-function activity Imatani et al[11] identified expression of a 1.8 Kb Notch4 mRNA transcript encoding a truncated and activated Notch4 intracellular domain in two of eight breast cancer cell lines tested. These authors further demonstrated that expression of this activated form of Notch4 in the mammary epithelial cell line MCF-10A, enabled these cells to grow in soft agar. Another study found Notch activation, evidenced by N^{IC} accumulation and Hey1 over-expression, in a wide range of breast cancer cell lines.[12]

The first clue that Notch may be aberrantly activated in primary human breast cancer came from a study demonstrating increased expression of Notch1 in four breast tumors that over-expressed H-Ras.[13] The authors demonstrated that down-regulation of Notch1 led to reduced proliferation of Ras-transformed human cells and that abrogation of Ras signaling prevented up-regulation of $N1^{IC}$. These findings suggested that $N1^{IC}$ functions

as a downstream mediator of oncogenic Ras and is necessary to maintain the neoplastic phenotype of Ras-transformed cells.

MECHANISMS OF NOTCH ACTIVATION IN BREAST CANCER

Until recently, activating mutations in *Notch* genes were thought to be rare events in human cancers. However, in 2004 Weng et al[14] discovered that more than 50% of T-cell acute lymphoblastic leukemias (T-ALL) harbored gain-of-function mutations in two key locations within the Notch1 receptor. The locations containing activating mutations are the extracellular heterodimerization (13% of cases) and the C-terminal PEST (26%) domains; in 18% of cases both domains contain activating mutations. Prompted by this discovery Lee et al[15] surveyed *Notch*1-4 for somatic mutations in 48 lung, 48 breast, 48 colorectal and 48 gastric carcinomas. Unlike the findings in T-ALL, somatic mutations in *Notch* genes were extremely rare in solid cancers with only two such mutations detected; one potentially activating, truncating mutation was identified in *Notch2* in a breast carcinoma (2.1%) and one *Notch*3 mutation in a colorectal carcinoma (2.0%).

In breast cancer Notch is activated primarily through up-regulation of Notch ligand and/or receptor expression rather than through activating mutations within *Notch* loci. Indeed, Jag1 and Notch1 mRNA levels are variable between patients and are elevated in breast cancers with poor prognosis pathologic features such as the basal-like subtype and high grade.[16] Patients with tumors expressing high levels of Jag1 or Notch1 have a significantly poorer overall survival compared with patients expressing low levels of these genes; a synergistic effect of high-level Jag1 and Notch1 co-expression on overall survival is also evident.[16-19] Consistent with these findings, elevated expression of $N1^{IC}$ in ductal carcinoma in situ (DCIS) lesions predicts early recurrence.[20]

Although recurrent DNA copy number alterations (CNAs) are common in breast carcinoma, large-scale genomic analyses have failed to identify functional amplifications in Notch ligand or receptor loci that would explain elevated expression of these genes. The 6p21-25 locus, which contains at least 200 genes including *Notch4*, is amplified in 34% of TN breast cancers but concurrent Notch4 over-expression has not been observed in these cases.[21] In a study that identified a crucial role for Notch3-mediated signaling in Erb2-negative breast cancer cells, Yamaguchi et al tested for *Notch3* amplification in 118 primary carcinomas and discovered only a single case.[22] Therefore, to date there is no evidence that CNAs significantly contribute to cases of elevated Notch ligand/receptor over-expression in breast cancer.

Current evidence suggests that up-regulation of Notch ligands and receptors occurs mainly through transcriptional and posttranslational mechanisms. For example, as described above, oncogenic Ras induces Notch1 expression in mammary cancers. In addition, Ras can up-regulate the expression of the Notch ligand Dll1 and the γ-secretase component presenilin 1, both of which may contribute to Notch activation.[13] Murine models corroborate a role for Ras in Notch-induced mammary carcinomas with the demonstration that transformation of mammary epithelial cells by Notch4 requires Erk/MAP kinase and PI-3 kinase pathways downstream of Ras.[23] Additional evidence for Ras-Notch cooperation in the transformation of the mammary epithelium comes from the observation that the bitransgenic progeny resulting from MMTV-H-ras mice and mice transgenic for the Notch antagonist Deltex (MMTV-Deltex) yield significantly fewer tumors than does the MMTV-H-ras parent line.[10]

Both Hypoxia (through up-regulation of p66shc) and IL-6 can induce expression of Notch3 and Jag1 and activate Notch signaling in breast cancer cells.[24,25] In this context, Erk/MAP kinase signaling is required for p66shc/Notch3 to induce both Jag1 and the hypoxia survival gene, carbonic anhydrase IX. This process is thought to control self-renewal and hypoxia survival in mammary gland stem/progenitor cells.

The Wnt pathway may augment the level of Notch signaling components. Ectopic expression of Wnt-1 results in tumorigenic conversion of human mammary epithelial cells through up-regulation of Dll1, Dll3, Dll4, Notch3 and Notch4 and activation of Notch signaling.[26]

Notch ligands and receptors undergo posttranslational modification in a context-dependant fashion and this is required for Notch activation. For example, modifications to the Notch receptor are conducted by ADAMs (a disintegrin and metalloprotease) and γ-secretase. Both ligand and receptor are modified by Fringe glycosyl transferases. GSK-3β[27] and CDK8[28] kinases determine Notch receptor half-life. E3 ligases (Itch, C-Cbl, Sel-10 and Deltex) and Numb (in cooperation with Itch) promote ubiquitination and degradation of Notch receptors.[29-32] There is evidence that posttranslational modification of Notch ligands/receptors may play a role in regulating Notch signaling in breast cancer. From the known list of potential regulators of Notch, Ras-induced γ-secretase stabilization (discussed above) and Numb loss have been implicated in the progression of carcinoma of the breast. Numb loss has been identified in 50% of breast cancers and correlates with high grade tumors.[33] In six tumors studied, Numb level was inversely related to the degree of Notch activation and the growth inhibitory effects of Notch antagonists. Consistent with these findings, Numb-defective breast tumors show a more aggressive tumor phenotype and a poorer prognosis.[34]

More recently the prolyl-isomerase, Pin1 has been shown to enhance Notch1 activation in cancer.[35] Pin1 contacts phosphorylated Ser/Thr-Pro motifs in its substrates and catalyses *cis-trans* isomerization of the intervening peptide bond inducing a conformational change.[36] Rustighi et al have shown that Pin1 binds to Notch1 and potentiates its cleavage by γ-secretase. This action results in increased release of $N1^{IC}$, increased Notch1 transcriptional activity as evidenced by Hes1 up-regulation and enhanced tumorigenic activity in breast epithelial cell lines. Remarkably, Pin1 is a direct Notch1 target gene, thereby promoting its own expression in a positive feedback loop. Consistent with Pin1/Notch1 cross-talk in breast cancer, levels of these two proteins correlate in primary breast cancer.

To summarize this section, most of the available evidence suggests that aberrant Notch activity in breast cancer results from the activation of pathways that regulate Notch expression or action, rather than through mutational activation or amplification of Notch ligand or receptor loci.

MECHANISMS OF NOTCH-INDUCED TRANSFORMATION

Although evidence implicating Notch activity in breast carcinogenesis is accumulating in both animal models and in human cells, the underlying mechanism(s) of Notch oncogenesis remain to be clarified. Notch signaling is a key regulator of diverse cellular processes including stem cell (SC) maintenance, cell-fate specification, differentiation, proliferation, cell motility and survival. Perturbation of these activities is a hallmark of cancer and so pathologic Notch activation has the potential to influence cancer progression at multiple levels.

Notch in Mammary Stem Cells and Tumor Initiating Cells

The theory that cancer is driven by a subpopulation of tumor-initiating cells (TICs) is decades old.[37] In this model tumors are composed of a hierarchy of cell types beginning with TICs which can both self-renew and repopulate the tumor, analogous to the way in which somatic stem cells (SCs) sustain organogenesis. That TICs exist is gaining wide acceptance as they continue to be identified in different malignancies including acute myeloid leukemia, breast, brain, prostate, melanoma, colon, pancreas and tumors of the head and neck.[38-47] In breast cancer, TICs were originally identified as lineage-negative (lin^-) $CD44^+/CD24^{-/low}$ cells.[39] These cells can form tumors in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice when as few as 200 cells are transplanted into the cleared mammary fat pad. In comparison, not even 10,000 unselected cells are capable of the same. Tumors resulting from the transplantation of $CD44^+/CD24^{-/low}/lin^-$ cells contain a small minority of $CD44^+/CD24^{-/low}/lin^-$ cells with the bulk of the tumor comprising nontumorigenic, phenotypically distinct cells; evidence that the originally injected cells can initiate and maintain the tumor by undergoing asymmetric division to produce both self-renewing and nontumorigenic daughter populations. Additional work has identified the aldehyde dehydrogenase (ALDH)–positive subgroup of $CD44^+/CD24^{-/low}/lin^-$ cells as further enriched for TICs.[48] These cells represent approximately 1% of the tumor and are highly tumorigenic with only 20 cells required to initiate a new xenotransplanted tumor.

A role for Notch in TIC biology is predictable based on work implicating Notch in self-renewal of the normal mammary SC and/or progenitor cell compartments. Through the use of an experimental in vitro culture system in which putative mammary SCs are cultured in vitro within multicellular spheroids (so called "mammospheres"), Dontu et al have proposed a role for Notch in mammary SC renewal.[49,50] The self-renewal capacity of mammospheres, which may reflect SC self-renewal, is enhanced ten-fold when mammospheres are cultured in the presence of a synthetic peptide derived from the DSL (Delta-Serrate-LAG 2) domain, highly-conserved in all Notch ligands and capable of Notch receptor activation. Consistent with these findings, mammosphere self-renewal is inhibited by Notch4 blocking antibody or an inhibitor of γ-secretase (GSI).

Bouras et al have examined the role of Notch in mammary SCs by using a short-term culture system in which freshly sorted epithelial subpopulations are genetically manipulated in vitro prior to testing their repopulating capability in the cleared mammary fat pad of mice.[51] In these studies, Notch inactivation in the murine mammary SC-enriched $CD29^{hi}/CD24^+$ population,[52] increases fat pad repopulating frequency. On the other hand, constitutive Notch signaling results in expansion of the luminal progenitor population. In contrast to the conclusions drawn from mammospheres, these data imply a role for Notch in restricting mammary SC proliferation and amplification of the luminal progenitor cell pool. Clearly, the method of assaying putative SC or progenitor cell populations is of critical importance and may explain these discrepant findings. Nevertheless, it is apparent that Notch is required to repopulate precursor populations early in the mammary epithelial cell hierarchy (Fig. 1).

Based on methodology to propagate SC within mammospheres, Ponti et al have described in vitro culture of putative breast TICs as multicellular tumorspheres.[53] Analogous to the mammosphere-SC relationship, tumorspheres share the properties of TICs. Tumorspheres contain undifferentiated cells that are capable of self-renewal and the generation of cells within daughter tumorspheres that can differentiate along pathways

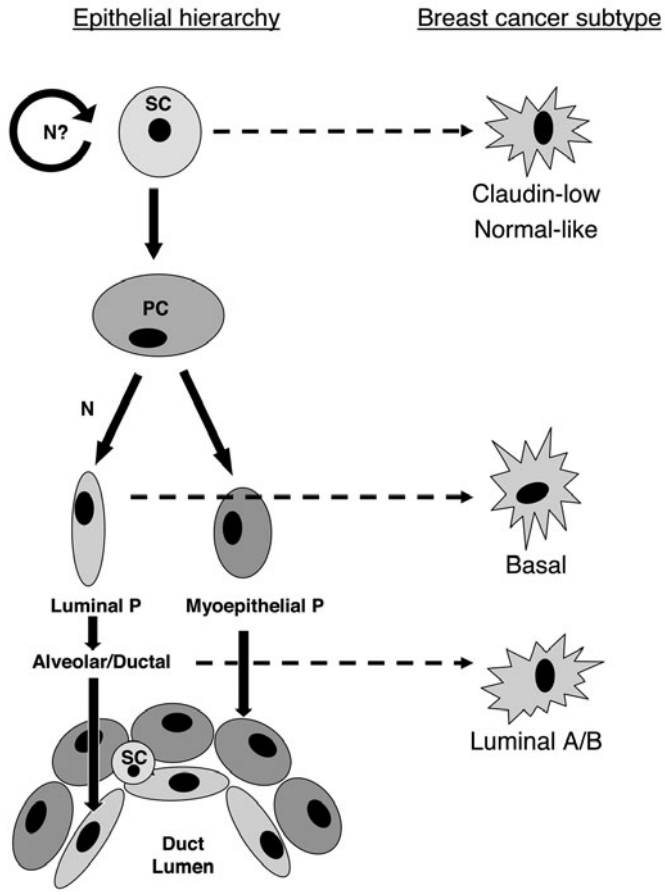


Figure 1. Notch and mammary epithelial cell hierarchy. Schematic diagram of mammary epithelial cell hierarchy demonstrating cell types that are possible targets for transformation to the indicated breast cancer subtypes (dashed arrow) based on gene expression profiling.[67] The role of Notch (N) in mammary stem cell (SC) maintenance is controversial (see text).[49-51] Notch activation promotes mammary progenitor cell (PC) fate decisions towards luminal differentiation (luminal progenitor, Luminal P)[51,65,66] and is associated with breast cancer of the basal subtype.[16,19,58]

to generate ductal and myoepithelial mammary lineages. Farnie et al have investigated Notch signaling in tumorspheres derived from ductal carcinoma in situ (DCIS).[20] These authors found that the efficiency of mammosphere production was significantly reduced when Notch signaling was inhibited either by GSI, anti-Notch4 monoclonal antibody or the anti-EGFR compound, gefitinib. These data suggest that Notch is required for DCIS TIC expansion. The findings also imply cooperation between EGFR and Notch signaling in TIC biology. There is also supporting evidence for a Notch/Her2 feedback loop (see below) which may maintain TICs in $Her2^{+}$ breast cancers.[54]

Using tumorsphere culture of primary breast cancers and breast cancer cell lines, Notch3 and Jag1 have been identified as a key regulators of TIC renewal and hypoxia survival.[24,25] Although promising, this method of in vitro propagation of TICs remains to be validated in further studies.

In addition to breast cancer, the Notch signaling network plays an important role in SC and TIC growth and proliferation in several other tissue lineages,[55] most notably in colon.[56] These findings hold promise that TICs may be therapeutically targeted through Notch antagonism. This hypothesis that inhibition of the Notch pathway can target and eliminate TICs in cancer must be tested molecularly and functionally in multiple tumor systems to fully validate its biological plausibility.

Notch, Lineage Specification and Breast Cancer Subtype

Breast cancer is a heterogeneous collection of diseases with approximately 60% expressing ER and/or PR (ER^+/PR^+) and approximately 30% over-expressing Her2 protein ($Her2^+$). For ER^+ and $Her2^+$ subtypes, effective targeted therapies include selective estrogen receptor modulators (SERMs) or aromatase inhibitors and trastuzumab, respectively. However, approximately 20% of breast cancer patients have TN disease and in spite of an initial response to systemic chemotherapy, their disease follows an aggressive clinical course with early recurrence.[57] The identification of targetable molecular pathways required for the growth of TN tumors will be of significant benefit.

Several lines of evidence suggest that Notch activation is associated with breast cancer of the TN subtype. For example, a statistically significant association between elevated expression of Notch ligands and receptors and TN/basal-like subtype has been demonstrated in resection specimens from breast cancer patients.[16,19,58] The association between Notch activation and up-regulation of the apoptosis inhibitor and cell cycle regulator Survivin, is exclusive to $ER^-/Her2^-$ basal-like breast cancer cells.[58,59] In a recent report examining the association between Notch activation and Her2 status, the importance of Notch-mediated signaling in the proliferation of $Her2^-$ and not $Her2^+$ breast cancer cells was shown.[22] Interestingly, in this study 4 of 5 $Her2^-$ breast cancer cell lines for which this association was established were also ER^-/PR^- (i.e., TN). Brca1-mutant breast cancers, which are predominantly ER^- and of the basal-like subtype, are associated with elevated Jag1 expression compared to their Brca2 (predominantly luminal) counterparts.[60] Consistent with these results, cell line data confirm an association between elevated Jag1 and the TN/basal-like subtype.[61]

Recent studies indicate that Notch may be activated in breast cancer cells where ER and Her2 signaling have been silenced. Estradiol treatment of ER^+ cells inhibits $N1^{IC}$ nuclear levels and Notch activity; these effects are in part mediated by inhibition of γ-secretase.[62] Treatment of ER^+ breast cancer cells with SERMs reverses this effect. Similarly, Her2 over-expression suppresses Notch1 activity in $Her2^+$ breast cancer cell lines and treatment with trastuzumab reactivates Notch signaling.[63] In fact, activation of Notch signaling may be a mechanism by which breast cancer cells become resistant to selective inhibition by trastuzumab. Taken together, these findings suggest that in the absence of the growth stimulatory effects of estrogen or Her2 (i.e.,TN or SERM-/herceptin-treated breast cancers), Notch activation may provide a compensatory, growth-promoting signal. These discoveries make the Notch signaling pathway an attractive therapeutic target in TN or drug-resistant ER^+ or $Her2^+$ breast cancers (discussed below).

While an association exists between Notch and TN/basal-like breast cancer, it is not clear whether Notch activation influences the development of tumors of this subtype. This possibility is worth exploring as Notch has a well-established role in lineage specification. For example, during hematopoiesis, Notch orchestrates expansion of the T-cell compartment from a common pluripotential progenitor.[64] By analogy, in the breast Notch biases mammary progenitor cell fate decisions towards luminal differentiation (Fig. 1). This was first suggested in a mouse model where Cre-mediated deletion of RBP-Jk or Pofut 1 (both required for canonical Notch signaling) in mammary progenitor cells, resulted in proliferation of basal cells and the accumulation of basal clusters during pregnancy.[65] The authors proposed a role for Notch in maintaining luminal cell fate at the cost of uncontrolled basal cell proliferation during alveolar development. In support of these findings, Raouf et al compared the transcriptomes of highly purified subpopulations of epithelial cells from normal human breast tissue to uncover a requirement for Notch3 during the commitment of bipotent progenitors to the luminal lineage.[66] These findings fit with the aforementioned work of Bouras et al indicating a role for Notch in amplifying the luminal progenitor population in the mouse mammary gland.

A recent study in humans comparing the relative abundance of epithelial subsets between breast tissue from BRCA1 mutation carriers and normal females, has advanced our understanding of the subpopulation of cells that may be the target of transforming events that eventually give rise to the TN/basal breast cancer subtype.[67] Lim et al found that compared to normal breast, preneoplastic breast tissue from BRCA1 carriers contains an expanded luminal progenitor population. The authors also showed, based on gene expression profiling, that breast tissue from BRCA1 carriers and basal breast cancers were similar to the luminal progenitor population but not to the basal stem/progenitor or mature luminal cell subsets. These data implicate the luminal progenitor population as a target for transformation in BRCA1-associated and possibly basal breast cancers in general (Fig. 1). Tying these findings together with data demonstrating a role for Notch in expanding the luminal progenitor compartment and evidence that Notch activation is associated with basal breast cancer, suggests a model whereby abnormal Notch activation contributes to the expansion of an aberrant luminal progenitor population, eventually giving rise to basal-like carcinoma.

NOTCH TARGET GENES AND TUMOR PROGRESSION

Proliferative and Anti-Apoptotic Effects of Notch Activation in Breast Cancer

In addition to promoting survival and lineage specification of breast cancer progenitor populations, Notch may also promote the malignant behavior of more "mature" populations in the tumor cell hierarchy. The involvement of Myc in the development and progression of breast cancer has been known for many years. More recently, Klinakis and colleagues used microarray analyses to compare the gene expression patterns of tumors derived from different transgenic mouse models in which the MMTV promoter was driving expression of an oncogene.[68] These authors noted that MMTV/*N1^{IC}* and MMTV/*Myc* mammary gland tumors exhibited a high degree of expression profile similarity and differentially expressed known Myc transcriptional target genes. Furthermore, conditional ablation of Myc in the mouse mammary epithelium of MMTV/*N1^{IC}* mice significantly reduced tumor frequency and increased tumor latency. Detailed molecular analyses revealed that the

Myc gene is a direct transcriptional target of Notch1 in mouse and human cells (Table 1). Immunophenotyping of human breast cancers demonstrated a statistically significant association between N1 and Myc expression supporting the existence of a functional relationship between these oncogenes in breast carcinogenesis.

Cyclin D1, a cell cycle regulatory protein required for advancement through the G1 phase of the cell cycle, plays an important pathogenetic role in breast cancer.[69,70] It is apparent from several studies in nonmammary tissues that the cyclin D1 gene (*CCND1*) can be regulated by Notch[71-74] and there is substantial evidence that *CCND1* is an important Notch target in breast cancer. Transgenic mice that over-express either H-Ras or Notch1 in the mammary gland develop mammary neoplasms with up-regulated cyclin D1.[10] Demonstrating the importance of cyclin D1 in these tumors, *CCND1* null mice fail to develop H-ras-induced mammary carcinoma.[75] In human breast cancer, the *CCND1* gene is a direct transcriptional target of Notch1 and Notch3.[61] Accordingly, Jag1 down-regulation reduces cyclin D1 expression and inhibits cell cycle progression through the cyclin D1-dependant G1/S checkpoint. Furthermore, cyclin D1 and Jag1 expression correlate in TN breast cancer expression datasets suggesting a model whereby Jag1 promotes cyclin D1-mediated proliferation of TN breast cancers. Additional cell cycle regulatory proteins, including cyclin A and B1, have been implicated as targets in Notch-mediated cell cycle progression in breast cancer.[62]

Akt has been shown to provide resistance to a numerous apoptotic stimuli. The recognition that Notch can regulate apoptosis in several contexts prompted Meurette et al to determine whether Akt was a mediator Notch-induced apoptotic resistance.[76] Indeed, normal breast epithelial cells require Akt activation for Notch-induced protection against apoptosis. In breast cancer cells Notch inhibition decreases Akt signaling and increases sensitivity to DNA damage-induced apoptosis.

Survivin, a member of the Inhibitor of Apoptosis gene family, functions as a regulator of mitosis and apoptosis and is over-expressed in a wide spectrum of malignancies including breast.[77] Interestingly, Notch signaling results in direct transcriptional up-regulation of Survivin, enhanced proliferation and increased cell viability in MDA MB231 cells.[59] Conversely, antagonism of Notch signaling in these cells results in reduced Survivin levels, apoptosis, reduced colony formation in soft agar and a defect in localized and

Table 1. Direct Notch target genes in breast cancer

Gene	Function	References
CCNA	Cell cycle regulation	62
CCNB	Cell cycle regulation	62
CCNDI	Cell cycle regulation	61
Hes1	Transcriptional repressor	63
Hey1, Hey2, HeyL	Transcriptional repressor	12, 80
Myc	Transcription factor	68
Pin1	Prolyl-isomerase/ post-translational modification	35
Slug	Cell migration/transcription	80
Survivn	Inhibitor of apoptosis	59

metastatic tumor growth in immunocompromised mice. In support of a biologically relevant connection between Notch and Survivn, a meta-analysis of multiple human breast cancer microarray datasets has revealed a statistically significant relationship between high Notch1 expression levels, increased Survivin expression and poor overall survival.[58]

Notch Activation, EMT, Invasion and Metastases

Epithelial-mesenchymal transition (EMT) is a process that contributes to the establishment of the body plan during development in multicellular organisms. An example of this occurs during normal heart development where Notch promotes EMT through up-regulation of the Snail repressor.[78] Indeed, this process is critical to proper formation of the cardiac valves. Under pathological conditions, EMT may promote invasion and dissemination of malignant cells. For example, over-expression of $N1^{IC}$ can promote mesenchymal transformation and EMT through Snail induction. These data suggests a role for Notch in promoting metastases through EMT.

Diverse evidence suggests that EMT is involved in the progression and dissemination of breast cancer cells.[79] Recently Slug, a zinc-finger transcriptional repressor, was identified as a direct Notch target in breast cancer cells.[80] Slug, like Snail, is known to represses E-cadherin expression and induce of EMT.[81] Leong et al have shown that Jag1 and Slug expression correlate in primary breast cancer and that blocking Notch activation in xenografted breast cancer cells restores E-cadherin expression, inactivates β-catenin and blocks growth and metastases in a Slug-dependant fashion. These results suggest that ligand-induced Notch activation promotes EMT and metastases in breast cancer, through a mechanism that is mediated by Slug.

Notch Signaling and Breast Tumor Angiogenisis

A role for Notch in physiological and pathological angiogenesis has been recognized for many tissue types (reviewed in Chapter IV.V). Vascular endothelial growth factor is a well-characterized inducer of tumor angiogenesis which can regulate tumor endothelial expression of Dll4.[82] Blockade of Dll4-Notch interaction results in decreased tumor growth as a result of a paradoxical increase in nonproductive tumor blood vessels. In a mouse xenograft model of the human breast cancer cell line MCF7, tumors express high levels of Dll4 in the mouse vasculature, supporting a role for Dll4-Notch signaling in breast tumor angiogenesis.[83]

In head and neck squamous cell carcinoma, Jag1 expressed in tumor cells promotes Notch activation and vascular network formation in neighboring endothelial cells.[84] Induction of Jag1 and Notch1 in MCF7 cells similarly results in vascular networking in adjacent endothelial cells.[85] In addition, primary breast cancer blood vessels express high levels of Notch3, implicating this receptor in breast cancer angiogenesis.[86]

NOTCH INHIBITION IN THE CLINICAL SETTING

As evidence for Notch activation in breast cancer continues to accumulate from cell culture experiments, mouse models and analyses of human tumors, strategies to target Notch in the clinic are emerging in parallel. To be successful, these strategies must

take into account Notch cross-talk with other signaling pathways such as estrogen- or Her2-mediated pathways.

Although the majority of breast cancers are ER^+ and can be treated with SERMs or aromatase inhibitors, resistance to these agents in many patients eventually manifests as local-regional recurrence or as a terminal metastatic event. Cross-talk between Notch and estrogen signaling is complex. As described above, estrogens inhibit Notch activity, whereas anti-estrogens or estrogen withdrawal activate Notch. Notch signaling, in turn, can activate ER-dependent transcription, suggesting the existence of feedback mechanisms controlling Notch-estrogen cross-talk.[87] From a therapeutic standpoint, these data indicate that combined inhibition of estrogen and Notch pathways may be effective in treating ER^+ breast cancers.

Breast cancers with Her2 over-expression are associated with aggressive disease and susceptibility to trastuzumab. Several clinical trials have demonstrated that in combination with chemotherapy, trastuzumab significantly improves overall survival.[88] However, most women with metastatic $Her2^+$ breast cancer display either de novo or acquired resistance to trastuzumab.[89,90] Evidence for an important interplay between Notch and Her2 exists. Notch regulates CBF-1-mediated transcription of the *Her2* gene[91] and an apparent negative feedback loop exists where Her2 over-expression suppresses Notch1 activity.[63] Predictably, trastuzumab treatment increases Notch activity in breast cancer cultures. Furthermore, trastuzumab-resistant breast cancer cell lines demonstrate activated Notch signaling and Notch inhibition in these cells restores trastuzumab sensitivity. These findings suggest that Notch1 activation plays a role in trastuzumab resistance and support the combination of Her2- and Notch-targeted therapies as a rational therapeutic approach.

Among Notch pathway inhibitors, small molecule GSIs have the most immediate therapeutic potential.[92] Highly selective and potent GSIs have been available for more than a decade and were originally developed as inhibitors of the γ-secretase complex that cleaves β-amyloid peptides, known to play a role in plaque formation in Alzheimer's disease.[93] With the discovery of γ-secretase-dependant Notch1 mutations in T-ALL, GSIs quickly moved into the preclinical realm as anti-cancer agents, demonstrating activity in several human cancer models through induction of apoptosis and reduced cell proliferation.[94-96] Predictably, Notch-activated breast cancer cells cultured in vitro either as a monolayer culture[62,63,97] or as tumorspheres[20,98] demonstrate marked growth suppression and reduced Notch activity upon GSI treatment. Similarly, in vivo GSI treatment blocks Notch signaling and tumor growth in human breast cancer cell line xenografts[62] and in a Her2 transgenic breast cancer mouse model.[99]

Although GSIs have demonstrated exciting outcomes in vitro and in mouse models of breast cancer, it is important to keep in mind the potential untoward side-effects of these compounds as they move into the clinic. Notch is involved in the cellular physiology of multiple tissue and organ systems and the potential exists for vital organ dysfunction with GSI treatment. Indeed, Notch signaling is involved in gastrointestinal (GI) homeostasis[100] and treatment with GSI can result in severe cytotoxicity within the GI tract.[101] GSI-induced dysfunction of the hematopoietic, vascular and other systems may also be appreciated as dose-escalation trials begin.

Another potential cause for GSI-induced toxicity relates to the fact that GSIs lack specificity. In addition to all four Notch receptors, GSIs target multiple additional proteins including Notch ligands, ErbB4, syndecan, CD44, E-cadherin and various proteases.[102-105] The design of clinical trials, dosing regimes and the determination of therapeutic windows will have to be carried out with an eye towards unanticipated side-effects.

Several trials of GSIs in T-ALL and solid tumors have recently been undertaken. MK-0752 (Merck) is a potent, orally active GSI in clinical development. Phase 1 clinical trials with this drug are either underway or have been completed, including trials in advanced breast or other solid malignancies.[106] In these studies, MK-0752 inhibited γ-secretase with a 50% decrease in plasma levels of the γ-secretase substrate, $Abeta_{40}$ within four hours after ingestion of the first dose. The main side effects of continuous dosing included fatigue, diarrhea, constipation, nausea and abdominal cramping. Ongoing MK-0752 trials in breast cancer include a study exploring different dosing schedules, and a study of MK-0752 in combination with tamoxifen or letrozole to treat early stage breast cancer.[107] Outcome data, including response rates and the assessment of intratumoral Notch inhibition have not yet been reported.

GSI RO4929097 (Roche) has also entered clinical trials. This drug has been evaluated in two dosing regimens: days 1-3 and 8-10 every 3 weeks or days 1-7 every 3 weeks.[108] The drug was found to be well-tolerated with the most common adverse events being reversible fatigue, nausea, emesis, diarrhea, hypophosphatemia and rash. In this study, pretreatment IL-6 and IL-8 levels have emerged as possible response predictive markers and will be explored further Another study combines R04929097 with the hedgehog inhibitor GDC-0449 in advanced breast cancer.[109] This combination is based on interconnectivity between these two pathways and their potential collaborative roles in TIC self-renewal pathways.[110]

The complexity of the Notch pathway introduces opportunities for signal inhibition at points within the cascade other than the γ-secretase complex-mediated cleavage. Antibodies, soluble ligands or receptor decoys have been useful in the preclinical setting to inhibit receptor activation.[82,84,111] Inhibition of proteins such as the Fringes or ADAMs, critical to Notch receptor activation, are being considered as potential therapeutic targets.[112] The Notch antagonists Deltex and Numb offer additional points within the cascade to influence signaling. A general interest in developing delivery systems for therapeutic RNAi is certainly relevant to inhibition of the Notch pathway.[113] In addition, signaling pathways upstream of Notch are potential therapeutic targets. For example the Ras,[13] MAPK[24] and PI3K/Akt/PTEN[114] pathways can regulate Jag1 expression in some contexts and these signaling cascades are the focus of intense preclinical and clinical investigations to discover novel, effective cancer therapeutics.

In addition to GSIs, Dll4 antibodies show promise as clinically useful Notch antagonists.[82,115] In a mouse breast cancer model, in which Dll4 was neutralized with DLL4-selective antibody, deregulation of angiogenesis resulted in inhibition of tumor growth.[115] Of note, the effects of Dll4 blockade in this study were restricted to the tumor vasculature, illustrating therapeutic potential without significant side-effects. Dll4 antibodies are also effective in reducing TIC frequency in vivo.[116] However complementary work has raised important safety concerns with the demonstration that chronic Dll4 blockade causes pathological activation of endothelial cells and induces vascular neoplasms in multiple animal models.[117] Early-phase clinical trials testing Dll4-targetted agents are currently underway and may determine whether the side-effects of these agents will overshadow potential therapeutic benefits.[118,119]

CONCLUSION

Since the discoveries of Gallahan et al[2] more than two decades ago, significant progress has been made towards understanding the pathologic role of Notch in the development and progression of breast cancer and other malignancies. Consistent with its pleotropic effects in normal development and tissue maintenance, aberrant Notch signaling plays a role in human breast cancer at multiple levels including TIC regulation, restriction of differentiation, cell proliferation, inhibition of apoptosis and promotion of invasion and metastases. There is growing evidence that crosstalk between Notch and key signaling pathways such as estrogen and Her2-mediated signaling can promote tumorigenesis. There is compelling preclinical evidence that agents targeting the Notch pathway, including GSIs and Dll4-ablating therapy, may provide novel strategies for the treatment of breast cancer. Clinical trials underway have been designed to test the efficacy and safety of Notch pathway inhibition either as single agents, or in combination with existing or novel targeted therapies for breast cancer. These early trials are critical as we enter the era of personalized cancer therapy.

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CHAPTER 19

NOTCH SIGNALING AND MALIGNANT MELANOMA

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Abstract: Increasing evidence indicates that Notch signaling contributes to physiologic processes, including development, differentiation and tumorigenesis, either as a tumor promoter or suppressor depending on the cellular context, level of expression and cross-talk with other signaling systems. Notch signalling has been implicated in the regulation of self-renewal of adult stem cells and differentiation of precursors along a specific cell line in normal embryonic development and organogenesis. There is also evidence that signaling through Notch receptors regulates cell proliferation and cell survival in several types of cancer including malignant melanoma, with opposing results depending on the tissue context. Tumor progression/metastasis of malignant melanoma are complicated processes that require multiple cellular events, including cell proliferation, survival, migration and invasion. Notch signaling appears to be a promising system for new therapeutic targets for the treatment of melanoma and perhaps the prevention of melanoma metastasis.

INTRODUCTION

Increasing evidence indicates that Notch signaling contributes to physiologic processes, including development, differentiation and tumorigenesis, either as a tumor promoter or suppressor depending on cellular context and the level of expression and cross-talk with other signaling systems.[1,2]

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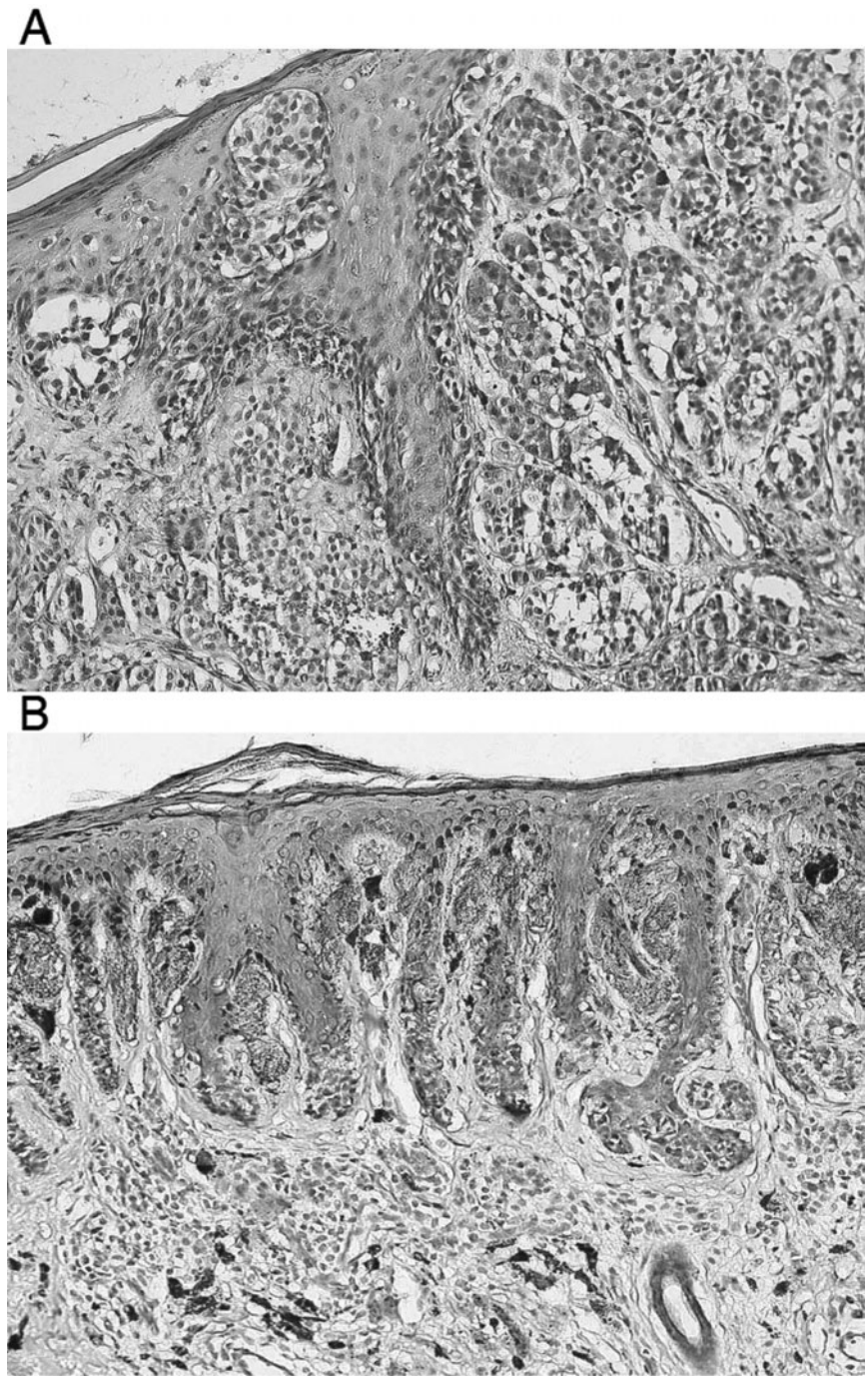


Figure 1. A) Expression of NOTCH-1 in invasive malignant melanoma. B) Expression of NOTCH-1 in common melanocytic nevi. Note that NOTCH-1 and -2 are expressed in malignant melanoma and melanocytic nevi, with distinct differences in these tissues.

PREFACE OF NOTCH SIGNALING

It is known that Notch signaling orchestrates the balance between differentiation and immature programs in suprabasal cells during epidermal development.[3] Notch and its ligands are abundantly expressed in the epidermis, where Notch signaling functions as a molecular switch that intervenes in cell transition between different skin layers during the epidermal differentiation process.[1] More recent findings obtained in melanoma and nonmelanoma skin cancers have shown that Notch signaling has a dual action (either as an oncogene or as a tumor suppressor), depending on the tumor cell type and the synchronous activation of other intracellular signaling mechanisms.[1]

NOTCH signaling is cell type-dependent and developmentally-regulated, underlining the relevance of other molecular pathways that modulate activity of this signaling pathway. NOTCH signaling depends on the presence or absence of several specific receptor proteins and corresponding ligands. In mammals, there are four transmembrane NOTCH receptors (NOTCH 1-4) which are activated via binding corresponding ligands of the Delta and Jagged families. It has been shown that in the canonical NOTCH signaling pathway, ligand-induced NOTCH receptor stimulation results in cleavage of the NOTCH intracellular domain, which then consecutively translocates to the nucleus and forms a ternary complex with the transcriptional co-activator, mastermind-like (MAML) protein, as well as the DNA-binding protein CSL, which has been shown to direct DNA-binding specificity and ultimately target gene expression. Until today, only a limited number of NOTCH target genes have been identified and characterized, the most important of which are the basic-helix-loop-helix proteins of the hairy and enhancer of split (Hes) and Hes-related transcription factor (Hrt) families, which function as transcriptional repressors.[4,5]

The Notch signaling pathway is an essential cell-cell interaction mechanism which regulates processes, such as cell proliferation, cell fate decisions, differentiation, or stem cell maintenance.[6] It has been suggested that the Notch signaling pathway is involved in tumorigenesis, as aberrant Notch signaling has frequently been observed in several cancers.[6] Depending on the cell type and context, Notch can either promote cell proliferation and cancer growth, or act as a tumor suppressor.[6,9,10] In keratinocytes, Notch signaling acts as a tumor suppressor and induces cell growth arrest and differentiation of squamous epithelia.[6]

Additionally, it is already known that aberrant Notch signaling might contribute directly to the pathogenesis of skin diseases, such as psoriasis, basal cell carcinoma and malignant melanoma and the Notch pathway is of great importance for the maintenance of proper hair pigmentation.[11] Recently, it was shown that general blockade of Notch signaling in tumor-bearing mice leads to defective angiogenesis in tumors, but depending on tumor cell types, general inhibition of Notch signaling might result in tumor regression, progression, or metastasis.[12]

NOTCH AND MELANOCYTES

Notch signaling is exceedingly important in melanocytes,[3,7,8] playing multiple roles in the melanocyte lineage,[8] thus interest in Notch signaling in melanocytic differentiation and melanoma pathology has become evident in recent years. Interestingly, expression of Notch-1 and -2, as well as Notch ligands, is up-regulated in dysplastic nevi and melanomas compared with common melanocytic nevi, indicating that activation of the

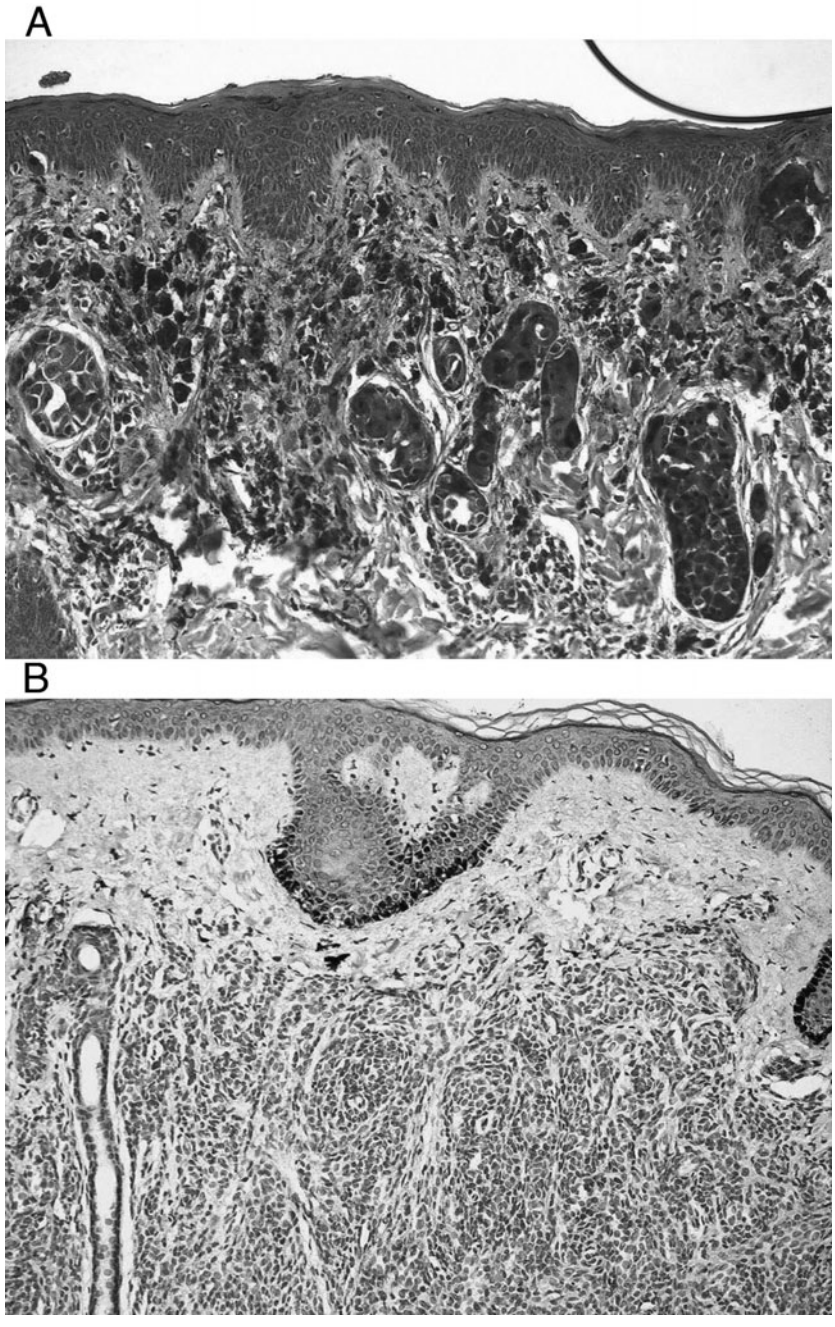


Figure 2. A) Expression of NOTCH-2 in invasive malignant melanoma. B) Expression of NOTCH-2 in common melanocytic nevi.

Notch signaling pathway may represent an early event in melanocytic tumor growth and up-regulation of Notch signaling may sustain tumor progression.[7]

NOTCH SIGNALING AND MALIGNANT MELANOMAS

Melanomas are highly aggressive neoplasms resistant to most conventional therapies and cause the majority of skin cancer-related deaths. Indeed, the incidence of melanomas is still increasing.[6] Melanoma development and progression is thought to be a step-wise process[6,13] that results from an interaction of altered intracellular tumor suppressors and oncogenes with the microenvironment in which these changes occur. It has been shown that Notch1 is a key effector of both Akt and hypoxia in melanoma development and the Notch signaling pathway could serve as a potential therapeutic target in the treatment of melanomas.[14] Notch signaling controls the cellular function of melanocytes by supporting the survival of immature melanocytes by preventing the initiation of apoptosis.[15] Inactivation of the Notch pathway leads directly to the loss of melanocytes[11] and causes a dramatic elimination of melanoblasts and melanocyte stem cells, which leads to a severe defect in hair pigmentation.[5,15] Depletion of RBP-J in mice leads to impaired hair pigmentation soon after birth and results in the subsequent progression of hair graying. RBP-J is a definitive downstream molecule required for Notch-signaling.[3]

It has been widely accepted that significant homeostatic regulation of melanocytes may occur through cell-cell interactions between keratinocytes and melanocytes.[16] A series of recent studies elucidated Notch signaling as a key component among keratinocyte-melanocyte interactions.[17] It has been shown that the Notch 1 pathway is activated in melanoma and suppression of Notch pathway activation inhibits melanoma growth in vitro.[18] Additionally, activated Notch 1 promotes human primary melanoma progression in vivo and increases β-catenin stability, specifically in primary melanoma cells[18] Balint et al[18] demonstrated that the oncogenic role of activated Notch 1 in promoting primary melanoma progression is β-catenin dependent. β-catenin is suggested as a functional target gene for Notch 1 signaling that mediates the tumor-suppressive effect in murine-skin carcinoma; in melanoma, however, β-catenin mediates the oncogenic role of Notch-1 signaling. The oncogenic role of Notch signaling in melanoma displays a stage-specific characteristic. Notch signaling advances primary melanoma, but has little effect on metastatic melanoma and appears insufficient to transform melanocytes on its own. Thus, specific collaborating genetic events are required for tumorigenic transformation of Notch signaling.[19] These stage-specific effects of Notch signaling in melanoma agree with the well-known temporal/spatial properties of Notch during development. Recently obtained consistent data show that primary melanoma cells are more sensitive to Notch-1 inhibition and MAP2 promotor up-regulation than metastatic melanoma cells.[20] Microtubule-associated protein-2 (MAP2) is a neuron-specific protein that stabilizes microtubules, is critical for neurite outgrowth and dendrite development and is frequently activated in human melanomas.[20] Melanoma, being a tumor originating from the neural crest-derived melanocytes, exhibits neural differentiation and aggressiveness of the tumor in vivo and is inversely correlated with MAP2 expression; overexpression of MAP2 in metastatic melanoma cells in vitro results in cell cycle arrest and growth inhibition.[20] However, in addition to the epigenetic influence, distinct metabolic components affect Notch signaling in melanocytes in vivo and in vitro. Müller et al[21] showed a distinct expression of NOTCH-1 and -2 and Jagged 1

in vivo in tissue slides of invasive malignant melanomas and benign common melanocytic nevi, as well as at the RNA and protein levels in vitro in MeWo cells. (Figs. 1 and 2) The results demonstrated cross-talk between vitamin D and NOTCH signaling pathways while regulating the growth of melanoma cells and it was concluded that both vitamin D analogs and pharmacologic modulation of NOTCH signaling may open new therapeutic perspectives for the treatment of malignant melanoma.[21]

CONCLUSION

The NOTCH signaling pathway has been shown to be of critical importance for the embryonic development and growth of human melanocytes. Tumor progression and metastasis is a complicated process that requires multiple cellular events, including cell proliferation, survival, migration and invasion. Notch signaling is a promising system for new therapeutic targets for treatment of melanoma and perhaps the prevention of metastasis.

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CHAPTER 20

NOTCH-SIGNALINGA ND NONMELANOMA SKIN CANCER: An Ancient Friend, Revisited

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Abstract: In humans and other species, Notch-signaling is of critical importance for carcinogenesis in several organs, including the skin. Interestingly, Notch-signaling appears to exert opposite roles in skin carcinogenesis as compared to carcinogenesis in other tissues. While the Notch1 receptor (Notch1) acts as a proto-oncogene in most tissues, it has been shown that Notch1 deletion in epidermal keratinocytes causes skin carcinogenesis. Recent results indicate that loss of Notch1 is not involved in the initiating event of multistage skin carcinogenesis, but acts as a skin cancer-promoting event. Moreover, recent findings underline the importance of multiple other factors, including the microenvironment, for Notch signaling in skin carcinogenesis. It can be speculated that pharmacologic modulation of Notch signaling may be an interesting target for the prevention and therapy of skin cancer.

INTRODUCTION

The Notch signaling pathway was first described as being responsible for ectodermal specification and neurogenesis in *Drosophila*.[1] In recent years, the Notch system has been demonstrated to be of critical importance for the embryonic development and the growth of various human cell types, including keratinocytes.[1-8] Moreover, it has convincingly been shown that Notch-signaling regulates a multitude of independent cellular processes, including differentiation, proliferation, angiogenesis, apoptosis and cell fate decisions, that are required for organogenesis and tissue homeostasis and that are involved in skin carcinogenesis.[1-12] Neoplasms originating from cutaneous epithelial cells, including

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basal cell carcinomas (BCC) and cutaneous squamous cell carcinoma (SCC), are the most common cancers in caucasian populations with annual incidences in the United States of nearly 1 million and 250,000 cases, respectively.[13-16] Unlike BCC, SCCs are biologically aggressive, arise from precursor lesions (actinic keratoses) and display metastatic potential with frequencies approaching 12.5%.[16] Mutations in components of the sonic hedgehog (SHH) signaling pathway have been implicated in the etiology of both human and mouse BCC.[17-20] By contrast, genetic mutations uniquely causing human SCC have not been identified,[19,20] although UV-induced mutations of the p53 gene are of particular importance for the photocarcinogenesis of SCC. Understanding the role of Notch signaling in BCC and SCC is of high relevance, given the enormous differences in the pathobiology and clinical outcome of these cutaneous malignancies.

NOTCH SIGNALING

Notch-signaling is evolutionary highly conserved, cell type dependent and developmentally regulated. A schematic demonstration of the Notch signalling pathway is shown in Figure 1. The relevance of other molecular pathways that modulate activity of this signaling pathway has been demonstrated.[21] It has been shown that the Notch system regulates developmental processes though binary decision, lateral inhibition and boundary formation.[22] Notch-mediated cell-cell communication is accomplished by coordinated, differential expression of Notch receptors and corresponding ligands on the cell surface.[1,2,7] In mammals, four evolutionary conserved transmembrane Notch receptors (Notch1-4) have been identified, that are activated via five ccorresponding Notch ligands of the *Delta* (Delta-like 1, 3 and 4) and *Jagged* (Jagged1 and 2) families (Fig. 1). In general, neighbouring cells stimulate each other to produce elevated levels of ligands and this effect then results in increased activation of Notch receptors. In most cases, increased expression of ligands with subsequent Notch activation causes cellular differentiation (and cell growth arrest), thereby regulating the cluster size of cell populations.

At the molecular level, it has been shown that in the canonical Notch-signaling pathway, ligand-induced Notch receptor stimulation results in cleavage of the Notch intracellular domain (NICD). NICD then consecutively translocates to the nucleus and forms a ternary complex with the transcriptional coactivator, mastermind-like (MAML) protein, as well as the DNA-binding protein, CSL, which has been shown to direct DNA-binding specificity and target gene expression.[23,24] Until today, only a limited number of Notch target genes have been identified and characterized, most importantly basic-helix-loop-helix proteins of the hairy and enhancer of split (*Hes*) and Hes-related transcription factor (*Hrt*) families, which function as transcriptional repressors.[25]

THE NOTCH SIGNALING PATHWAY IN THE SKIN

Notch receptors and corresponding ligands are present in the skin, although most of their particular functions are at present still uncertain.[4] It has been shown that Notch receptors and ligands are differentially expressed in the different cell layers of the viable epidermis.[26] In healthy skin Notch1, Delta1 and Jagged1 are present in all cell layers of the viable epidermis, with pronounced expression of Delta1 and Jagged 1 in the basal layer. It has been observed that Delta/Notch signaling is increased in cells that undergo a

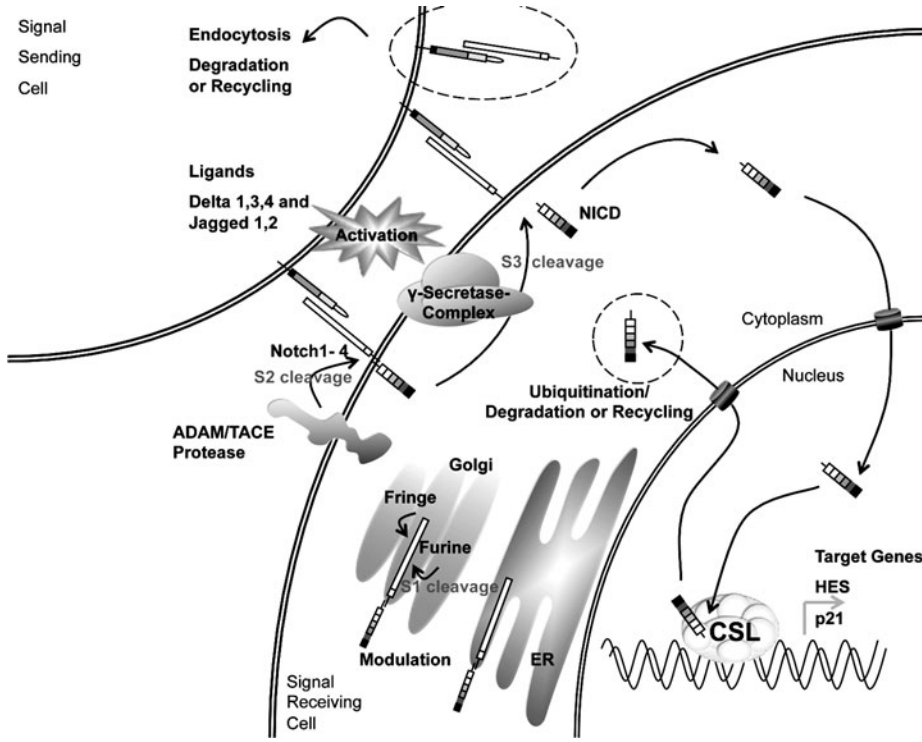


Figure 1. Notch signaling represents an evolutionarily highly conserved pathway in multicellular organisms that regulates cell-fate decisions through juxtacrine signaling among adjacent cells during development, in stem cells and in cancer cells. In mammals, four different Notch receptors (Notch 1-4), that are present in signal-receiving cells and that activated by binding to corresponding ligands in signal-sending cells, have been identified so far. They represent single-pass trans-membrane receptor proteins composed of functional extracellular (NECD), transmembrane (TM) and intracellular domains (NICD). In the signal-receiving cell, endoplasmatic reticulum (ER) and Golgi processing of Notch receptors causes cleavage, thereby producing a glycosylated, Ca^{2+}-stabilized heterodimer composed of NECD noncovalently attached to the TM-NICD inserted in the membrane (S1 cleavage). This processed receptor protein then translocates to the plasma membrane for binding to a corresponding ligand. In mammals, members of the Delta-like (DLL1, DLL3, DLL4) and the Jagged (JAG1, JAG2) families, which are in general present in the signal-sending cell, function as ligands that can activate corresponding Notch receptors. Following ligand binding, the NECD is cleaved (S2 cleavage) from the TM-NICD domain by the ADAM metalloprotease TACE (TNF-α converting enzyme). The NECD remains bound to the ligand. This protein complex is then processed by endocytosis and recycling/degradation within signal-sending cells. In the signal-receiving cell, a third cleavage event mediated by γ-secretase releases the NICD from the TM (S3 cleavage), which then translocates to the nucleus and associates with the CSL family transcription factor complex, thereby causing activation of individual Notch target genes, including Myc, p21 and HES family members. In most cases, increased expression of ligands with subsequent Notch activation causes cellular differentiation (and cell growth arrest), thereby regulating the cluster size of cell populations.

normal differentiation program, as in cell layers of the normal adult human epidermis.[26] In contrast, Notch signaling has been described to be decreased in hyperproliferating skin conditions, including psoriasis vulgaris.[26] In line with these investigations, it has been reported that loss of Notch1 in young mice induces hyperproliferation of the basal epidermal layer and deregulates expression of multiple differentiation markers, including

expression of p21 that is reduced and of Gli2 that is elevated.[5,9] In epidermal keratinocytes, activated Notch1 has been shown to induce p21 expression in a CSL-dependent manner, resulting in cell cycle withdrawal and terminal differentiation.[5] In addition, Notch1 directly promotes caspase 3 activity, that is required for terminal differentiation of embryonic keratinocytes.[6] However, the physiological/pathophysiological function and the regulation of the Notch system members in the pathogenesis of human skin tumors are not yet completely understood.

NOTCH SIGNALING AND NONMELANOMA SKIN CANCER

In accordance with its function in inducing differentiation of keratinocytes, mice with an experimentally-induced epidermal deletion of the Notch1 gene were shown to exhibit extensive epidermal hyperplasia and spontaneously develop BCCs.[9] Consequently, this finding has resulted in the hypothesis that Notch1 may act in the skin as a tumor suppressor.[9,27] Moreover, in mice with epidermal inactivation of Notch1, chemical injury promoted the formation of cutaneous lesions representing both BCC and SCC, in addition to inducing numerous papillomas.[9] It has been shown that mice expressing a dominant negative MAML1 (DNMAML1) protein to inhibit CSL-dependent Notch signaling in the epidermis exhibit multiple skin defects including diffuse alopecia, epidermal hyperplasia and hyperkeratinization. These mice develop spontaneous lesions resembling human SCC and actinic keratoses, but do not develop BCC. In contrast to normal epidermis, keratinocytes and lesional cells from DNMAML1 mutant mice express nuclear ß-catenin and cyclin D1 in a pattern similar to that observed in human cutaneous SCC, suggesting a conserved role for these molecules in SCC. Taken together, these data strongly suggest that functional interactions between Notch signaling and ß-catenin and cyclin D1 play critical roles in the pathogenesis of cutaneous SCC.

Figure 2 shows the immunohistochemical detection of Notch1 in human BCC (**A**) and SCC (**B**). As outlined above, animal models have shown that epidermal skin tumors that spontaneously develop in mice lacking Notch1 display basal cell carcinoma (BCC)-like phenotype. In line with these findings, it has been shown that in BCCs, the protein expression of Notch receptors and corresponding ligands, Delta1 and Jagged1 is markedly lowered in tumor regions[26] as compared to healthy epidermis. Interestingly, Thélu and coworkers also reported that they were unable to detect these proteins in the regions with pallisading cells penetrating the dermis. In summary, an increasing body of evidence indicates that, in absence of Notch1, Delta1 and Jagged1, missing or decreased Notch signaling leads to disorder in epidermal differentiation and proliferation[26] and promotes formation of BCCs. Impaired Notch signaling is also reported to promote the development of cutaneous squamous cell carcinoma (SCC), as outlined above and malignant melanoma (MM).[28,29] In summary, it can be assumed that in contrast to other tissues, Notch seems to function in the skin as a tumor suppressor, as shown by Nicolas et al.[9]

As reported above, the physiological/pathophysiological function and the regulation of the Notch system members in the pathogenesis of human skin tumors are not yet completely understood. Nevertheless, it has been speculated that pharmacologic modulation of Notch signaling could be a new promising target for the treatment of skin cancer. Recently, it has been demonstrated that topical treatment with immunomodulatory-acting agent imiquimod selectively up-regulates gene expression of Notch receptors (Notch1) and ligands (Jagged1, Delta1) in BCC.[30] Imiquimod represents a strong immune response

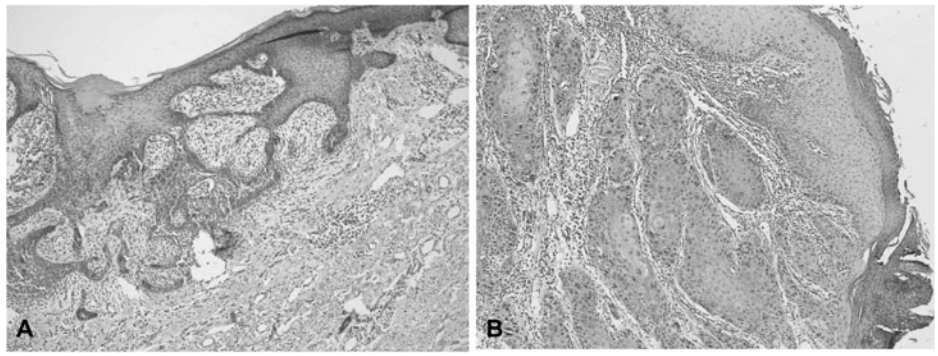


Figure 2. Immunohistochemical staining patterns of Notch1 in paraffin sections of BCC (A) and SCC (B). Please note that in unaffected epidermis, cytoplasmic and nuclear staining (red labelling) for Notch1 is seen in basal cell layers (A,B). Both in BCC and SCC, tumor cells are stained for Notch1 as well.

modifier that exerts its effects via activation of toll-like receptor 7 (TLR7), thereby resulting in an activation of NF-κB,[31] increased synthesis of proinflammatory cytokines and a potent stimulation of anti-tumor Th1 immunity.[32,33] Interestingly, Jagged1 is implicated to represent a downstream target of NF-κB activation providing a link between these two signalling pathways.[34] As outlined above, Notch1 and Jagged1 play an important role in the differentiation of keratinocytes and the activation of Notch signaling promotes terminal differentiation of epidermal keratinocytes.[2-6]

THE EFFECTS OF NOTCH1 DELETION ON MULTISTAGE EVENTS IN SKIN CARCINOGENESIS

As outlined above, it has been shown convincingly that Notch1 deletion in epidermal keratinocytes causes skin carcinogenesis, while in contrast Notch1 acts in most other tissues as a proto-oncogene.[35] Recently, the mechanisms underlying the carcinogenesis-promoting characteristics of Notch1-deficient skin have been analyzed in mice with a global or chimeric deletion pattern in their epidermis.[36] Results of this study obtained by deleting Notch1 either before or after DMBA treatment in the *K14CreERT* system indicate that loss of Notch1 is not involved in the initiating event of multistage skin carcinogenesis.[37] However, it was shown that Notch1 loss acts as a skin cancer-promoting event. In this study, delaying Notch1 deletion in K14CreERT mice until after the tumor-promotion stage of carcinogenesis demonstrated that late deletion of Notch1 contributed to malignant progression of benign papillomas, a phenotype that is observed upon loss of p53 but not loss of $p21^{WAF1/Cip1}$,[38] a specific Notch1 target in the skin.[5] In summary, the authors concluded that the main effect of Notch1 loss in skin carcinogenesis is to provide the initiated cells with a proliferative signal to promote tumor growth and proceed to invasive skin cancer. It has been speculated that this proliferative signal is located downstream of Notch1 loss and could be originated from within the initiated cells, supporting Notch1's role as a classical tumor suppressor in epidermal keratinocytes.[9] As an alternative pathway, it has been hypothesized that this signal could be delivered by the skin microenvironment reacting to Notch1 loss in the epidermis.[39-42] The experimental system used by Demehri et al[36]

allowed to distinguish between these two possibilities. In their study, the chimeric pattern of Notch1 deletion by *Msx2-Cre* created neighboring territories of Notch1-expressing and Notch1-deficient keratinocytes coexisting in the same microenvironment. Examining a large number of tumors isolated from DMBA/TPA-treated Msx2-N1CKO mice clearly demonstrated that tumors comprised mostly (>99%) of Notch1-expressing cells were as likely to form as tumors comprised predominantly of Notch1-deleted cells in the same environment. The authors concluded that Notch1 loss in the epidermis generates a noncell autonomous signal, promoting tumorigenesis from any initiated cell exposed to the microenvironment conditioned by Notch1-deficient keratinocytes. These results underline the relevance of the microenvironment as an active contributor to tumor development[43] by demonstrating that it can be the primary source of proliferative signals to initiated cells.

CONCLUSION

Notch signaling governs tissue development during embryonal organogenesis, while in adult tissues it contributes to maintenance of cellular differentiation, proliferation and apoptosis, modulates stem cell functions and plays a role in skin carcinogenesis. Recent findings demonstrate that Notch signaling has in nonmelanoma skin cancer a dual action (either as an oncogene or as a tumor suppressor), depending on the tumor cell type and the synchronous modulation of other intracellular signaling mechanisms. In this review, we summarize the role of the Notch signaling pathway in normal skin homeostasis and differentiation and focus on its altered regulation in the tumorigenesis of BCC and SCC. Further understanding of the pleiotropic roles of Notch signaling in BCC and SCC may provide a rationale for identifying Notch as a new therapeutic target for the treatment and/or prevention of these malignancies.

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CHAPTER 21

NOTCH SIGNALING AND INTESTINAL CANCER

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Abstract: In recent years, a substantial body of evidence has accumulated to support the notion that signaling pathways known to be important during embryonic development play important roles in regulating self-renewing tissues and tumorigenesis. In this context, Notch signaling is now recognized as essential for maintaining progenitor/stem cell population as well as for regulating cell lineage differentiation in the normal intestinal mucosa. Many studies have also showed that Notch signaling is constitutively activated in colorectal cancer and its inhibition is able to suppress the cell growth and sensitize cancer cells to treatment-induced apoptosis. Therefore, discovery of the role of γ-secretase in the Notch signaling activation has prompted intensive research on the potential use of γ-secretase inhibitors in the treatment of colon cancer. This chapter reviews the current understanding and research findings of the role of Notch signaling in intestinal homeostasis and colorectal cancer and discusses the possible Notch targeting approaches as novel molecular therapy for intestinal cancer.

INTRODUCTION: ADULT INTESTINAL AND COLONIC CELL HOMEOSTASIS

The mammalian intestine is a paradigm of rapid and continuous tissue renewal, as its turnover rate outpaces all other tissues in the body, likely contributing to its susceptibility to malignant transformation.

The gut resembles a tube containing two anatomically and histologically distinguisable major structures, the small intestine and the colon, which are respectively involved in digestion/absorption of nutrients and compaction of stools.

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During early embryonic development, the visceral endoderm appears uniform and presents stratified cell layers. Intestinal epithelial cytodifferentiation occurs during fetal development and is marked by mesodermal growing into the lumen and formation of finger-like protrusions called villi (Fig. 1). These villi are separated by the proliferative intervillus epithelium, which is reshaped downward in the mesenchyme forming deep invaginations know as crypts of Lieberkühn. The formation of these crypt-villus structures relies on reciprocal signaling between the endoderm and the mesoderm (EM).[1,2] In human, final architecture of the small intestine is reached before birth and is characterized by the crypt-villus unit, which allows for a great increase in surface area for absorption of nutrients. The colonic villi disappear at the time of birth and the mature colonic epithelium present tubular glands (crypts). Consequently, the adult intestinal wall is covered with numerous crypts and villi in the small intestine, in contrast to a flat surface epithelium in the colon (Fig. 1).

In each small intestinal crypt, around 4-6 stem cells reside within an optimal environment called « niche », that includes epithelial and mesenchymal cells and extracellular substrates.[3-5] Asymmetric division of stem cells give rise to transient undifferentiated cells that proliferate vigorously and are found exclusively in the crypts. They are believed to represent the first stage of specialization into the four cell types present in the intestine. These cells either belong to (i) the absorptive cell lineage, such as enterocytes that represents up to 80% of all epithelial cells and are responsible for degradation and absorption of nutrients and (ii) the secretory cell lineage, including the hormone secreting entero-endocrine cells, the mucus secreting goblet cells and the antibacterial peptide producing Paneth cells (Fig. 1). As progenitor cells migrate upward and exit the crypt, they enter the villus compartment, stop dividing, acquire their differentiated functions and are finally extruded into the gut lumen (a process called exfoliation), with exception of Paneth cells that always are located at the bottom of the crypts. Luminal migration of crypt progenitors is both (i) passive due to being "pushed" by newly "born" progenitors from the crypts, apoptotic loss of cells from the villous tip and (ii) active from newly described epithelial cell-cell and EM signals. Therefore, in the small intestine, the crypts are mainly occupied by undifferentiated cells whereas the villi contain terminally differentiated cells. The colonic epithelium is similar to that of the small intestine, except for the absence of villi and Paneth cells (Fig. 1).

This well-compartimentalized structure is suitable for analysis of developmental processes ; however, study within this tissue has been limited until very recently by lack of a robust in vitro culture system for primary intestinal epithelial cells.[6,7] Studies with transgenic and knockout mice have shed light on molecular mechanisms underlying the fetal development of intestine as well as the homeostatic epithelial regeneration in the adult, which requires a tight regulation of proliferation, differentiation, migration and cell shedding. As the adult intestinal epithelium may be viewed as a "developmental" system, several developmentally critical pathways, that control self-renewal and cell lineage commitment, including Wnt, TGFβ/BMP Hedgehog and Notch, have provided insight into the mechanisms that underlie homeostasis of the intestinal mucosa and intestinal cancer.[8,9] More recently, the Notch signaling pathway has been implicated as a key determinant of intestinal epithelial self-renewal, differentiation and cancer.

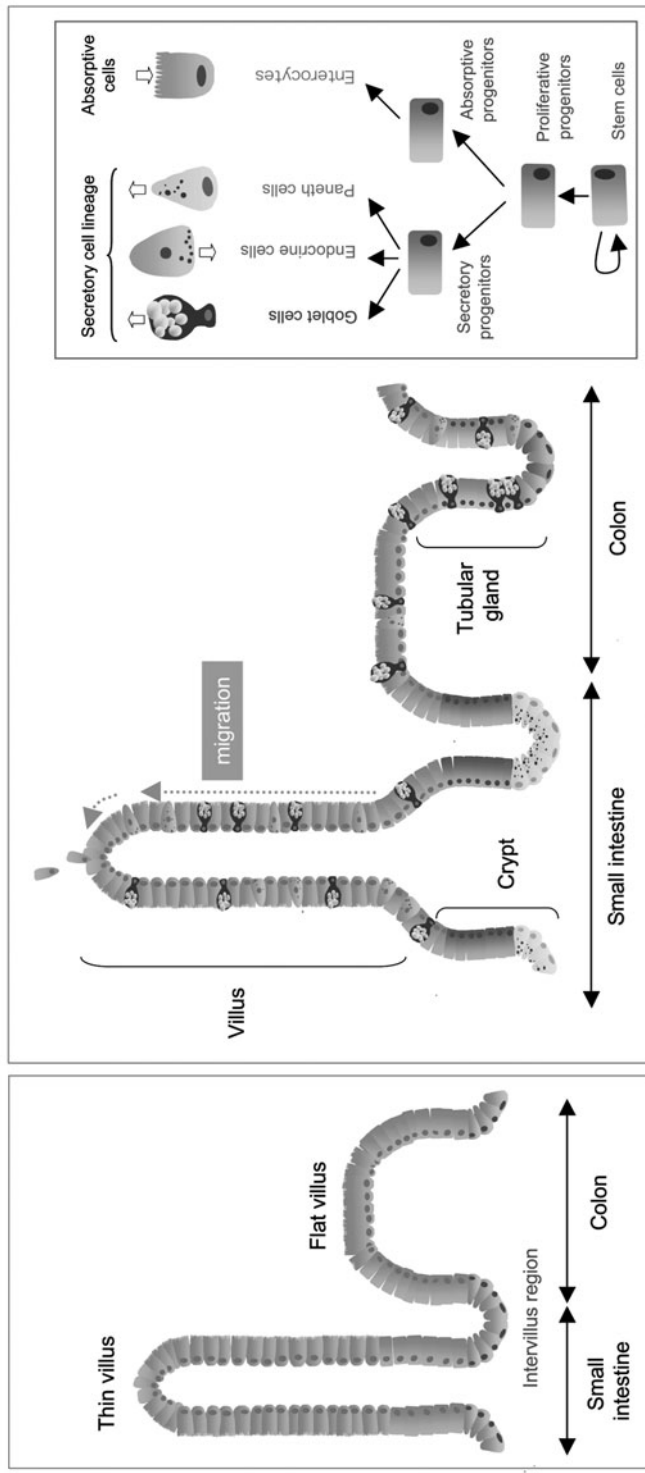


Figure 1. From development to differentiation of the intestinal epithelium. During the early embryonic development, the gut endoderm appears as a stratified epithelium. Intestinal epithelial cytodifferentiation occurs during fœtal development and is marked by mesodermal growing into the lumen and villi formations. These villi are separated by proliferative intervillus epithelium. Anterio-posterior axis differences appear and are characterized by long and thin villi in the small intestine and by transitory wide and flat villi in the colon. The intervillus epithelium of the small intestine is reshaped downward forming crypts. In adult intestine, the final architecture, which is characterized by the crypt-villus axis, is reached before birth. In the crypt, the stem cells are located in position +4, just above the Paneth cells, and give rise to both secretory and absorptive progenitors, which are responsible for generating the mature cell types within their respective lineages.

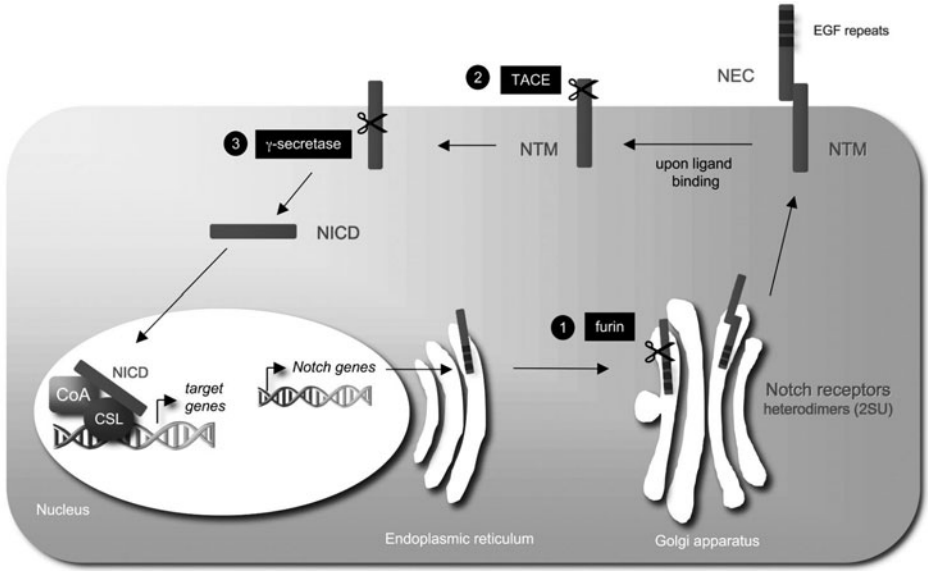


Figure 2. Proteolytic processing of Notch receptors. Notch is a large cell-surface receptor that is activated by contact with membrane-bound ligands on neighboring cells. Three different proteolytic steps process the 300 kD precursor of mature Notch. One protease is a furin-like enzyme that cleaves Notch constitutively adjacent to the amino acid sequence RQRR in the extracellular domain, which give rise to heterodimeric Notch receptors composed of the Notch extracellular subunit (NEC) and the Notch transmembrane subunit (NTM). Binding of extracellular ligand to Notch induces two other proteolytic steps: Notch processing is first carried out by TACE, also known as ADAM17, and the subsequent cleavage at the transmembrane region involves a γ-secretase activity that is dependent on presenilin-1. Cleavage of Notch in the extracellular domain releases the Notch intracellular domain (NICD) which migrates into the nucleus where it associates with CSL transcription factors and acts as a transcriptional coactivator.

THE BASICS OF NOTCH SIGNALING

Notch genes were first characterized in 1917 by Thomas Hunt Morgan as a stain of *Drosophila* exhibiting notches at the end of their wing blades due to haplo-insufficiency of the Notch gene. The Notch signaling network has been known for decades to developmental biologists as a key player pattern formation and in cell fate determination in all eukaryotes. The four mammalian Notch genes encode single-pass transmembrane receptors (called Notch1 to Notch4) that are activated by cell-membrane-associated ligands belonging to Jagged (also known as Serrate) and Delta-like families, therefore participating in communication between contiguous cells.[10] The interaction of Notch-Delta interaction mediates a phenomenon called "lateral inhibition" whereby a single cell that is beginning to differentiate prevents its neighbors from differentiating in the same way at the same time.

Mature Notch molecules derive from a golgian proteolytic processing of precursors that are cleaved by a furin-like protease into heterodimers comprising an extracellular subunit (NEC) noncovalently associated with a transmembrane subunit (NTM) (Fig. 2). NEC contains multiple EGF-like repeats, which are involved in

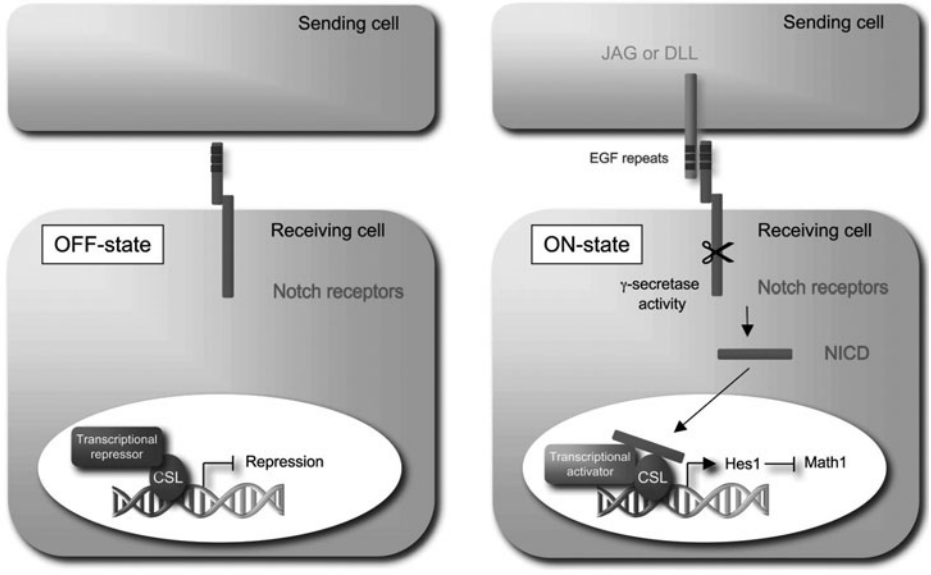


Figure 3. Notch, a membrane-bound transcription factor. In the absence of Notch signaling, the DNA binding protein CSL acts as a transcriptional repressor. Upon binding to their ligands Jagged (JAG) or Delta-like (DLL), Notch receptors are cleaved by an enzymatic complex with a γ-secretase activity. This proteolytic step results in the release of the Notch intracellular domain (NICD), which subsequently enters the nucleus where it binds to CSL. As a results, CSL converts from a transcriptional repressor to a transcriptional activator. The outcome is that the downstream target genes, such as that encoding the repressor Hes1, are upregulated, which in turn repress expression of downstream genes (e.g. *Math1*).

ligand binding, while NTM participates in CSL (also known as CBF1, Suppressor of Hairless or Lag-1) binding and ubiquitination. Notch hetedimers dissociation upon ligands binding causes a two-step proteolytic cleavage; first, the extracellular portion of NTM is clipped by TNF-α converting enzyme (TACE), a metalloprotease of the A disintegrin and metalloprotease (ADAM) family. This makes NTM susceptible to an additional cleavage in the transmembrane domain by a presenilin-1-dependent γ-secretase activity (Fig. 2). This last cleavage releases NICD (Notch Intracellular Domain) from the plasma membrane in the cytosol. The generation and stability of NICD is regulated by several E3 ubiquitin ligases, which influence the intensity and duration of Notch signals.[10]

NICD translocates to the nucleus where it subsequently activates the expression of downstream target genes by binding to a highly conserved transcription factor, CSL (a collective name of CBP or RBP-Jκ in vertebrates, Su (H) in *Drosophila* and Lag-1 in *Caenorhabditis elegans*). The quaternary complex formed by NICD, CSL, DNA-binding protein and transcriptional coactivators of the mastermind-like (MAML1) family associates with specific regulatory DNA containing CSL-binding sequences and recruits additional factors (such as p300 and PCAF) with histone acetylase activity. In the absence of NICD, CSL can recruit repressor complexes to the cis-regulatory region of the CSL-Notch target genes. Therefore, activation of Notch triggers a switch from repression to activation (Fig. 3).

DISTRIBUTION OF NOTCH SIGNALING COMPONENTS IN THE GUT

Among many other target genes, the expression of the HES (Hairy Enhancer of Split) basic helix-loop-helix (bHLH) transcriptional repressor family is regulated upon Notch activation. In particular, NICD stimulates the expression of Hes1,[11] that in turn represses the activity of others bHLH transcription factors, including Math1 (Fig. 3).[12,13]

The distribution of Notch receptors and their ligands in the gastrointestinal tract has been described in rodents and human.[14-17] More specifically, it appears that Notch ligands, Notch receptors and some downstream components-such as *hes1* and the transcription factor CSL- are expressed in the gut epithelial layer. Notch1 receptors activation is restricted to the intestinal crypt compartment in mouse and human,[15,18,19] consistent with the expression of Hes1 in the crypt progenitors in embryo or adult mice.[14] Reciprocally, Math1 is expressed in goblet cells, entero-endocrine cells and Paneth cells in the gut but does not colocalize with all ki67-expressing progenitors, suggesting that Math1 expression is essential only for cells that have just exited cell cycle.[13,20] Moreover, Notch signaling appears to be inactive in precursors of the secretory cell type, allowing for the expression of Math1.

Interestingly, the expression of the Notch ligand Dll1 is confined in a small fraction of crypt progenitors and in differentiated goblet cells. [17, 21, 22] In accordance with the lateral inhibition model, Dll1-positive cells may trigger Notch signaling in neighboring cells and force them to adopt an enterocyte fate. A rather similar distribution was observed for Dll4 in human neoplastic goblet cells[23] whereas controversial studies report Jagged1 expression either in the villi in entero-endcorine cells or in the crypt.[24,25]

NOTCH AND GUT CELL FATE DETERMINATION

The Notch pathway was initially implicated in the regulation of cell fate decisions in the intestine after mice deficient for two putative Notch target genes *hes1* or *math1* were produced.[13,14]

Analysis of the developing fetal intestine of *hes1*$^{-/-}$ mice revealed a striking increase in the number of different entero-endocrine subtypes and goblet cells together with a decrease of intestinal enterocytes, suggesting that Hes1 signaling controls allocation of cells to a secretory fate in the gut.[14] Among the target genes of Hes1-mediated repression, *math1* is essential for cells to make the first secretory lineage-specifying choice. In fact, the intestinal mucosa of Math1-deficient mice show a relatively normal crypt-villus architecture that is populated entirely by enterocytes and devoid of goblet, Paneth and entero-endocrine cells.[13,26] This phenotype is somewhat reciprocal to the phenotype observed in *hes1*$^{-/-}$ mice, indicating that Hes1 and Math1 are able to skew the fate of differentiating cells exiting from the transit-amplifying compartment towards either an enterocyte or a secretory phenotype. Taken together these results suggested that Notch signaling regulates a binary decision between absorptive and secretory cell fates (Fig. 3 and Fig. 4).

This intestinal secretory cell fate inhibition by Notch receptors was also suggested in vivo by the use of γ-secretase inhibitors (GSI), such as dibenzazepine. GSI were first developed as an attractive approach for the treatment of Alzheimer's disease to reduce the production of pathogenic β-amyloid peptides resulting from the cleavage of the amyloid precursor protein. However, other proteins have been shown to be substrates for

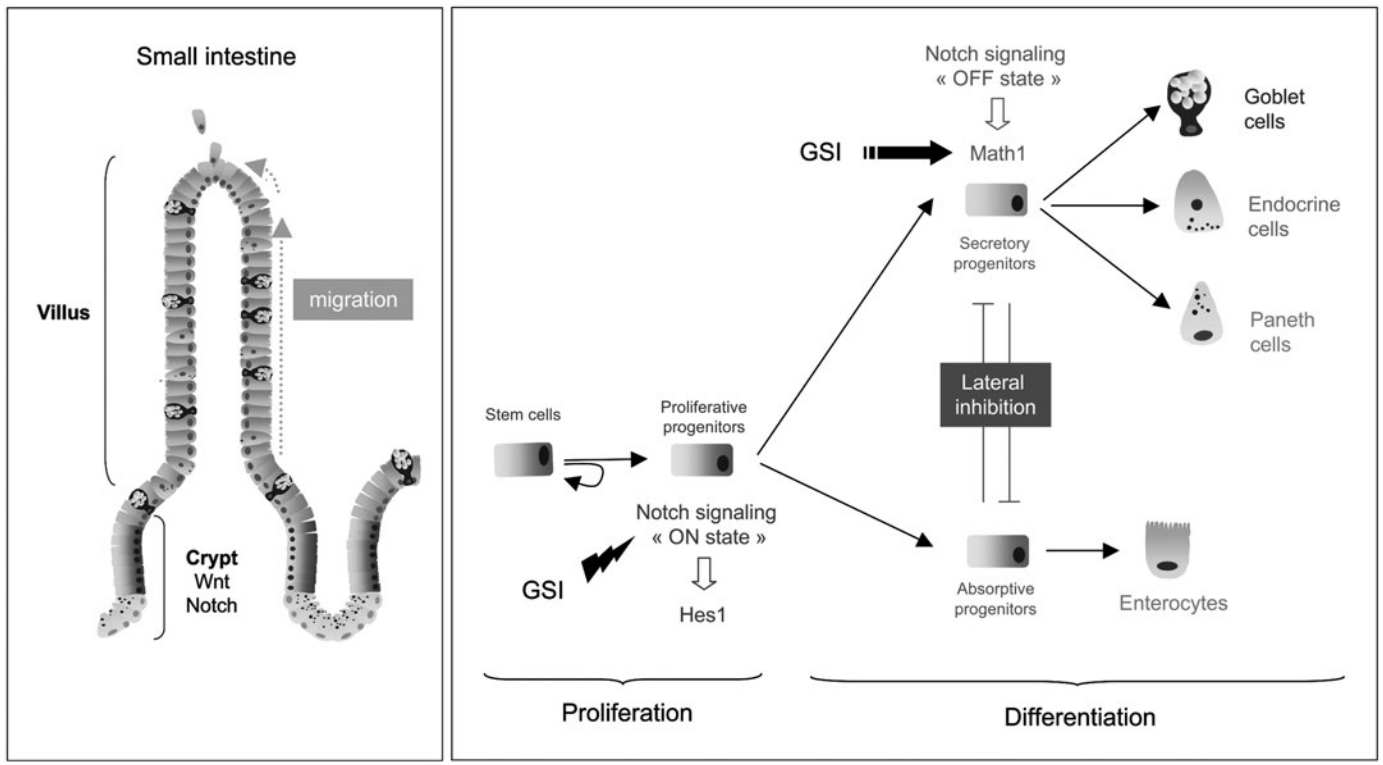


Figure 4. Notch signaling pathway and intestinal cell commitment. Stem cells (in red) and proliferative progenitors (in blue) localize in the crypt compartment, which is maintained by both Wnt and Notch signaling pathways. One function of Notch signaling within the small intestine is to maintain proliferative crypt progenitors in the undifferentiated state, while a second function is to influence a binary cell fate decision of proliferative progenitors that have to choose between the absorptive and the secretory lineages such as goblets cells, Paneth cells and entero-endocrine cells. This process seems to be regulated by the Notch target gene *Hes1*, which transcriptionally represses Math1. Math1 expression is required for the development of secretory lineages. A color version of this figure is available at www.landesbioscience.com/curie.

γ-secretase cleavage, including Notch receptors. Thus, in rodents, chronic injections with GSI caused a goblet cell metaplasia and an upregulation of secretory cell type markers (somatostatin, mucin, CCK, gastrin) expression.[27-29]

The ultimate demonstration of a direct role for Notch signals in controlling the segregation of each mature lineage from undifferentiated progenitors cells, as well as maintenance of the proliferating intestinal pool, came from the analysis of mice carrying either a loss- or gain-of-function.[18,30] Postnatal inactivation of the Notch pathway by conditional deletion of the *csl* gene (that mediates Notch signaling of all Notch receptors) results in the loss of Hes1, in the consequent de-repression of the *math1* gene and in the abundant ectopic expression of nuclear Math1 protein in the crypt compartment (whereas it is normally restricted to secretory cells). Immuno-histochemical analysis of the intestine of these mice revealed a complete loss of proliferative cells, that are converted into postmitotic goblet and entero-endocrine cells, at the expense of enterocytes.[18,27-29] Further mechanistic insights into how Notch signaling regulates intestinal homeostasis came from inducible intestinal-specific loss-of function approaches. Notch1 and Notch2 receptors appear to act redundantly in the gut, as only simultaneous loss of both receptors results in complete conversion of proliferating crypt progenitors into postmitotic goblet cells, similar to mice with gut-specific inactivation of the *csl* gene.[18,31] Interestingly, the expression of a constitutively activated form of Notch1 receptor (NICD1) in the intestinal epithelium, markedly impairs differentiation of crypt progenitor cells and increases the proportion of dividing cells, which extend outside of the crypt proliferative compartment.[30] This Notch-mediated maintenance of the proliferative crypt compartment in the small intestine requires normal Wnt signaling[32] and involves transcriptional repression of two cell cycle inhibitors $p27^{kip1}$ and $p57^{kip2}$.[31] Taken together, these reciprocal loss- and gain-of-function experiments demonstrate that Notch receptors function redundantly as gate-keepers for intestinal crypt progenitor cells to maintain their proliferation and to control their binary cell fate decision lineage specification (Fig. 4).

NOTCH-MEDIATED LATERAL INHIBITION PROCESS

Regarding the implication of the Notch ligands in proliferation and differentiation of the intestinal epithelial cells, lineage tracing in mice has shown that cells expressing Dll1 differentiate toward the secretory lineage, whereas knock down of *dll1* in human colonic epithelial cells completely abrogated the GSI-induced up-regulation of *muc2* (a goblet cell marker) expression.[17] This suggests that induction of the goblet cell phenotype in the colonic epithelium is dependent upon the up-regulation of *dll1* expression. In contrast, in vivo studies have shown that the administration of a neutralizing antibody for another Notch ligand Dll4 had no effect on murine intestinal epithelium.[33] Further experiments in human colonic epithelial cells have demonstrated that expression of *dll1* and *dll4* are regulated by the Notch/Hes1 axis. In fact, forced expression of NICD1 or Hes1 over-expression results in a significant down-regulation of *hath1* (the human orthologue of *math1*), *dll1* and *dll4* expression and suppress the goblet cell phenotype of the human colonic cells LS174T,[17,34] whereas treatment with GSI induces *hath1*, *dll1* and *dll4* expression in LS174T and HT29 cell lines.[34] A reciprocal increased expression of *dll1* was reported in intestine of $hes1^{-/-}$ mice.[14] Thus, the complementary expression of NICD and Delta-like ligands suggests that Dll-Notch feedback regulation, a prerequisite in lateral inhibition, may be operating within the colonic epithelium (Fig. 5).

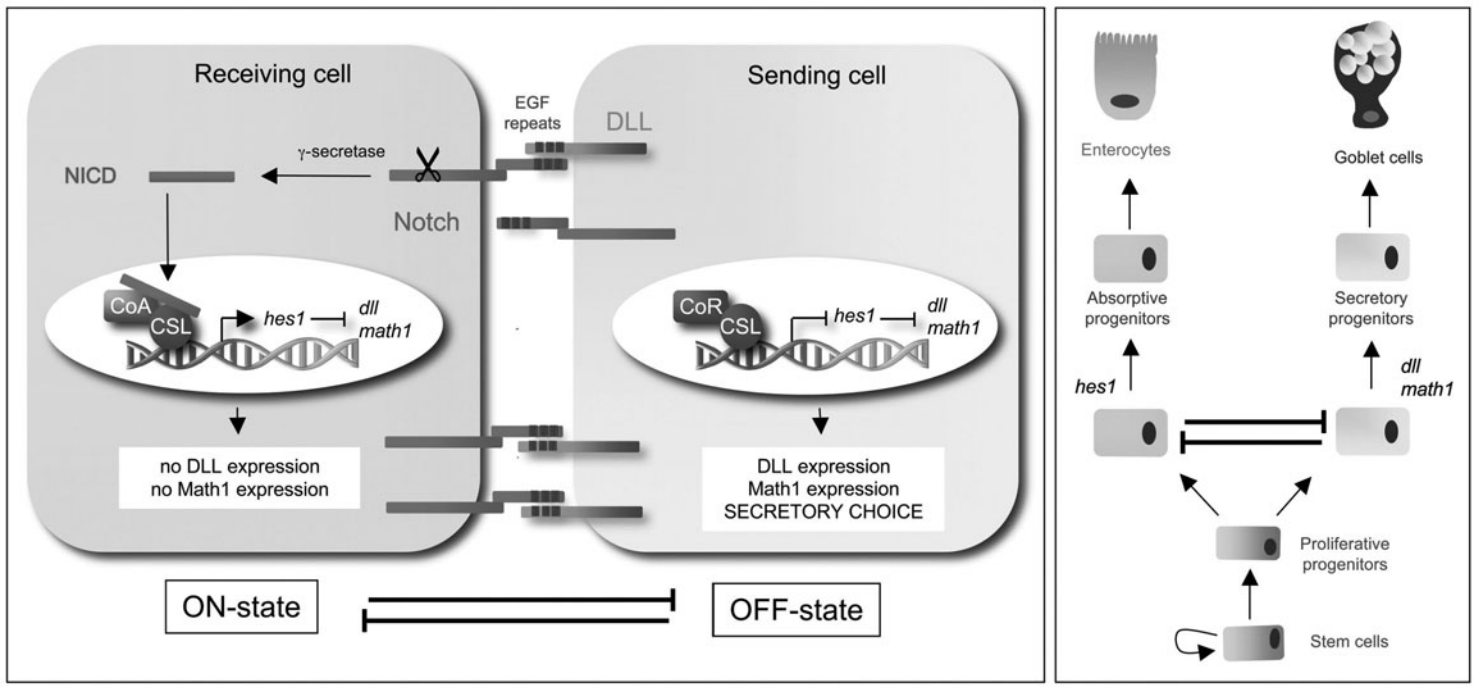


Figure 5. Delta-Notch mediated lateral inhibition. Math1, a helix-loop-helix transcritpion factor dowstream of the Notch signaling pathway, regulates intestinal epithelial cell fate decisions. In crypt progenitor cells that express high levels of Notch, the Hes1 transcription factor is switched on whereas the expression of Math1 and Delta-like ligands is blocked, resulting in enterocytes differentiation. In sending cells, the weak activation of the Notch pathway, which results from the low Dll expression in neighboring receiving cells, blocks the production of Hes1, and subsequently induces Math1 and Dll expression, and secretory cell determination. Thus, production of the Math1 transcription factor allows precursor cells to make a choice: whether to become goblet cells, Paneth cells, or entero-endocrine cells. A color version of this figure is available at www.landesbioscience.com/curie.

Interestingly, the involvement of Notch signals in cell fate determination in the intestine seems to be conserved in all eukaryotes. In zebrafish, inhibition of Notch signals achieved by two different genetic lesions (the DeltaD and the *mindcomb* mutants) leads to an overproduction of secretory cells at the expense of absorptive cells, accompanied by an increase in apoptotic cells.[21] In addition, an important function for the Notch/Hes1 pathway in maintenance of intestinal stem cells, in control of proliferation and endocrine cell fate specification in the invertebrate gut of *Drosophila melanogaster* has been recently shown, further underscoring the essential role of Notch signaling in this organ.[35-39]

LINKS BETWEEN NOTCH AND COLORECTAL CANCER: WHAT DO WE KNOW?

Colorectal cancer, the second most prevalent cancer in the western world and the third most prevalent in developing nation is responsible worldwide for 11% of all cancer deaths annually.[40] Colorectal cancer develops through a stepwise process that involves a variety of genetic and epigenetic changes that are acquired over several years and culminate, eventually, in the transformation of normal epithelium to neoplasm.[8] Recent advances in treatment comprise variations on the classical themes of surgical resection combined with chemotherapy using cytotoxic drugs and radiation therapy. Because this therapy is only moderately successful, novel approaches to the treatment of colorectal cancer are required. Our rapidly increasing knowledge of molecular signaling pathways that are deregulated in colorectal cancer might provide a platform from which to develop new rational cancer therapies.

In this context, the oncogenic potential of Notch signals has been documented in a variety of experimental systems and in several tissues, as well as in intestinal tumorigenesis. A connection between Notch and intestinal tumors arises from studies showing that Hes1, a signature of Notch signal activation is expressed uniformly in mouse adenomas,[18,19,41] in human colorectal cancer (CRC) and colon cancer-initiating cells,[32,42-44] whereas *hath1*, the human orthologue of *math1*, is progressively down-regulated with increasing colonic tumor severity in CRC samples (Fig. 6).[20,45] Deletions and epigenetic silencing via methylation were shown to act in combination to cause loss-of-function mutations of the *hath1* locus in primary human cancer.[45] Interestingly, these observations of enhanced Notch activation in colorectal cancer suggest a strong symmetry of the expression pattern between crypt cell progenitors and intestinal neoplasia, as it was recently demonstrated by gene profiling experiments.[46] Therefore, the question whether proliferating adenoma cells can be differentiated and withdrawn from the cell cycle by inhibiting Notch signaling, similarly to what is observed with crypt progenitors, was investigated. Treatment of $Apc^{(min/+)}$ mice (a model of intestinal tumorigenesis) with GSI induces *math1* expression in adenomas, confirming the pharmacological inhibition of the Notch pathway. This is accompanied by the rapid conversion of proliferative adenoma cells into postmitotic goblet cells and a subsequent 50% decrease in the number of adenomas in the small intestine of $Apc^{(min/+)}$ mice, suggesting that the maintenance of the proliferative potential of adenoma cells depends on Notch activity.[18,47] In vitro, treatment of human colon cancer cells (HT29, HCT116 or LS174T) or isolated colon cancer-initiating cells (CCIC) with GSI reduces proliferation, induces apoptosis and secretory cell lineage differentiation whereas over-expression of NICD increased their rate of proliferation.[34,44,47] Similar results are obtained using small interfering RNA-mediated *csl* inhibition, demonstrating that

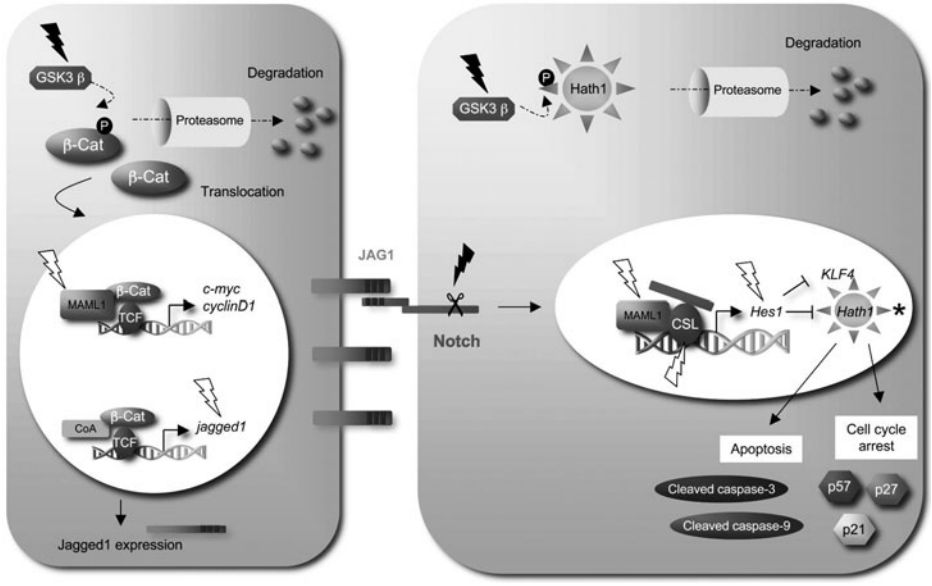


Figure 6. Simplified diagram illustrating Notch signaling in colorectal cancer cells and potential targeting approaches. Whites arrows indicate RNAi or antagonizing approaches whereas black arrows indicate pharmacological approaches (gamma-secretase inhibitors or GSK3-beta inhibitors). The asterisk indicates deletion and epigenetic silencing on *hath1* locus.

the canonical Notch pathway is critical to intrinsic maintenance of CCIC self-renewal.[44] However, Notch receptors act redundantly to maintain tumor growth in vivo, as Notch1 or Notch2 inhibition does not alter proliferation of colon cancer cells.[48]

Further examinations of the underlying mechanisms required for Notch-mediated progression of neoplastic growth have involved the tumor suppressor genes *hath1*[20,45] and *Krüppel-like factor 4 (klf4)*.[12,47,49] Conditional inactivation of *math1* in two different mouse models of colon tumorigenesis strongly enhances the formation and progression of colonic tumors, either induced by a chemical carcinogen (azoxymethane) or in $Apc^{(min/+)}$ mice, suggesting that loss of *math1* can be an initiating event in mammalian cancer formation.[45] Complementary studies in vivo and in vitro in HT29 cells have demonstrated that Math1 is a key mediator of anti-proliferative and pro-apoptotic effects, as it triggers a JNK-dependent induction of cell cycle inhibitors, such as $p27^{kip1}$ and $p21^{cip1/waf1}$ and caspase-3 and -9 cleavage (Fig. 6).[20,45] Consequently, over-expression of Hath1 in HT29 cells induces cell cycle exit and goblet cell differentiation, suppresses anchorage-independent growth in a soft agar colony formation assay and, more significantly, inhibits growth of HT29 cells in xenograft experiments.[20] In addition to Hath1, the effect of Notch pathway on intestinal tumorigenesis was reported to involve Hes1-mediated repression of the zinc finger-containing transcription factor KLF4 (Fig. 6).[12,47,49]

Taken together, available data from many studies have demonstrated that aberrant activation of Notch signaling plays an oncogenic role in colorectal cancer. These "proofs of principle" experiments highlight the Notch pathway as a potential drug target for the treatment of intestinal neoplasia. However, in strategies to target Notch signaling, it will be a challenge to find a therapeutic window for GSI, in which crypt function remains

sufficiently intact whereas neoplastic cells are forced to differentiate[18] and to avoid potential complications, such as alterations in the mucosal immune response, in strategies to target Notch signaling.[28,50]

REGULATION OF ANGIOGENESIS BY THE NOTCH PATHWAY

The progressive development of colonic tumors and subsequent metastasis are dependent on their "angiogenic switch" which stimulate neovascularization and subsequent supply with oxygen and nutrients. The major driving force of angiogenesis comes from some pro-angiogenic growth factors released by tumor tissues, among which the vascular endothelial growth factor (VEGF) is the most potent one. It is now known that overexpression of VEGF in colonic tumors correlates with angiogenesis, metastasis and poor prognosis in patients, demonstrating that anti-angiogenesis therapy is a promising strategy for colorectal cancer.

In this context, targeting of the Dll4 Notch ligand has been suggested as an attractive approach to control angiogenesis and thus indirectly restrain invasive colonic tumor growth and metastasis.[51] Despite some expression in neoplastic goblet cells in colonic tumors, Dll4 is mostly expressed by the endothelium of colon cancer cells and not in the endothelium adjacent to normal mucosa.[23] Blocking Dll4 with a selective neutralizing Dll4 antibody has been reported to induce hyperproliferation and defective cell fate specification of endothelial cells, thereby inhibiting growth of colonic tumor cells xenograft in vivo.[33] Importantly, Dll4-mediated Notch signaling appears to be crucial during active vascularization, but less important for normal vessel maintenance. Therefore, targeting Dll4 might represent a valuable new approach for colon cancer therapy, as it might help to avoid deleterious effects on the normal intestinal mucosa.

INTERACTION BETWEEN NOTCH AND WNT SIGNALING PATHWAYS

Functional Wnt signaling pathway is essential to control cell proliferation and tumorigenesis in the intestinal epithelium (Fig. 7).[52] Deregulation of this pathway initiates intestinal tumor development in many human cancers including colorectal cancer, as mutations in the *apc* gene have been found in most sporadic colorectal tumors and in those arising in FAP (familial adenomatous polyposis) patients. Aberrant Wnt signaling leads to the constitutive transcriptional activation of β-catenin/TCF target genes, associated with various important processes of tumorigenesis such as sustained cellular proliferation in the absence of growth signal.[53-55]

In this context, constitutive Notch activation alone does not cause intestinal tumor formation. However, Notch and Wnt signaling cascades were shown to act synergistically in intestinal tumor initiation, as transgenic expression of NICD1 in the intestine increases formation of intestinal and colonic adenomas in $Apc^{(min/+)}$ mice.[32] Notch activation was also linked to β-catenin-dependent tumorigenesis, as specific inhibition of the Notch pathway can drive intestinal tumor cells out of cell cycle despite the fact that Wnt signaling remains active.[20,32,51] Additional microarray analyses have provided insight into the mechanisms leading to Notch activation and the contribution of the Notch pathway to colorectal cancer. Notch was shown to be downstream of Wnt through β-catenin-mediated transcriptional activation of the

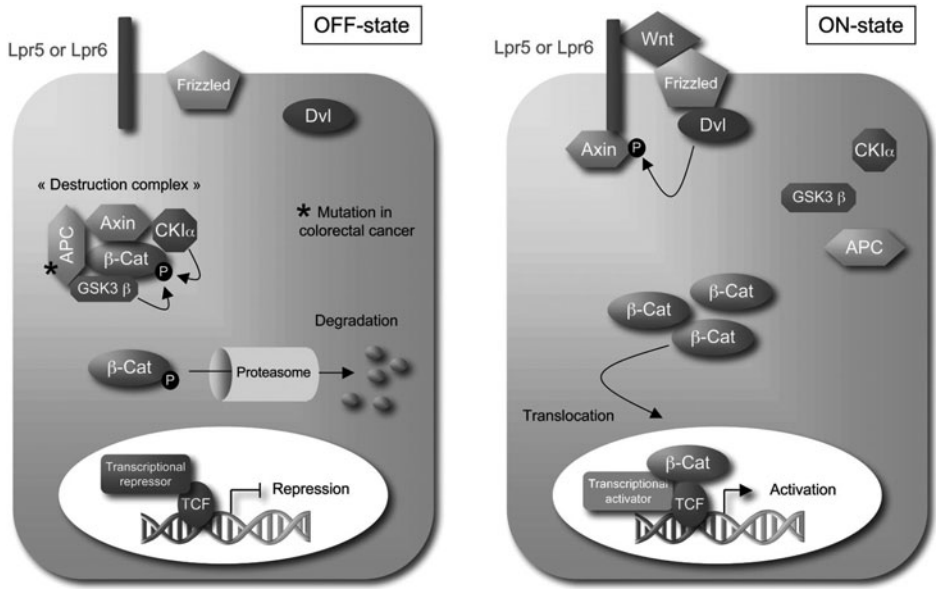


Figure 7. Wnt signaling pathway, a two states model. A general scheme for Wnt/β-catenin signaling has been established. The binding of Wnt to Frizzled and LDL cell-surface receptors (Lpr5 or Lpr6) initiates a cascade of signaling events, including the recruitment of Dishevelled (Dvl) and the subsequent inactivation of glycogen synthase kinase 3 (GSK-3). The inhibition of GSK-3 phosphorylation of β-catenin leads to the stabilization of cytoplasmic β-catenin and its translocation into the nucleus, where it interacts with the T-cell factor (TCF) family transcription factors, and converts them from repressors to activators. In the absence of Wnt signaling, β-catenin is sequestrated in the cytoplasm by a destruction complex containing adenomatous polyposis coli (APC), and axin. In this complex, β-catenin is phosphorylated by casein kinase I (CKI) and GSK-3. Phosphorylation of β-catenin by these kinases leads to ubiquination and degradation by the proteasome. This mechanism has evolved to tightly regulate the levels of β-catenin activity in the nucleus.

Notch-ligand gene *jagged1* (Fig. 6).[24,25,56,57] Consistently, in intestinal tumors arising from the constitutive activation of the β-catenin/TCF transcriptional complex in *Apc1638*$^{(N/+)}$ or *Apc*$^{(min/+)}$ mice, *jagged1* expression is heterogeneously up-regulated, thus leading to aberrant activation of Notch1 and Notch2 receptors. Inactivation of the *jagged1* gene in *Apc*$^{(min/+)}$ mice induces a reduction in the size of the polyps, whereas expression of activated Notch1 (but not the one of Hes1) partially reverts the effects of blocking Wnt/β-catenine pathway in colonic tumors xenografts in mice. This suggests that Notch is an essential modulator of tumorigenesis induced by nuclear β-catenin and that Notch-targets downstream of β-catenin, other than Hes1, participate in regulating colorectal tumorigenesis. Consistently, Rodilla et al reported an increased expression of Jagged1 in human tumors from Familial Adenomatous Polypsis patients, suggesting that partial inhibition of Notch in an active β-catenin background may be therapeutically relevant for FAP patients.[25]

Among the potential cross talk between Notch and Wnt signaling, two essential components (MAML1 and Hath1) of the Notch signaling pathway have been reported to be downstream of the Wnt regulatory network. In fact, MAML1, a transcriptional co-activator of CSL, is recruited in vitro and in vivo by β-catenin on Wnt target gene

promoters containing TCF-binding sites (for example, cyclin D1 and c-myc) and this interaction results in a dramatical increase of β-catenin transcriptional activity, even in the presence of GSI (Fig. 6).[58] These data indicate that MAML1 functions in the Wnt/β-catenin pathway independently of Notch signaling. Strikingly, the knockdown of MAML proteins in colonic carcinoma cells, SW480, resulted in cell death, which correlated with decreased expression of cyclin D1 and c-myc. Thus, reduction of MAML1 expression could be an alternative target for cancer therapy regulating both Notch and β-catenin-mediated transcription of target genes such as c-myc and cyclin D1.

Interestingly, Hath1 was also established as a common target of the Notch and Wnt activation cascades.[59,60] The first evidence of this interaction came from the observation of stabilized Hath1 protein and subsequent goblet cell differentiation after inactivation of Wnt signaling by forced expression of the full-length *apc* gene in colon cancer cells.[20,60] In vitro, GSK3β was shown to mediate both β-catenin and Hath1 protein degradation, suggesting that treatment with a GSK3β inhibitor have potential for use in a new therapeutic approach to induce differentiation of most colon cancer cells (Fig. 6).[59,60]

REGULATION OF NOTCH SIGNALING IN CHEMOPREVENTION AND CHEMOTHERAPY

Colorectal cancer is associated with a high mortality, due to the late stage at which many cases present. Attention is therefore focusing on preventative strategies for colorectal cancer given that polyps appear to be identifiable and treatable precursor lesions of this disease. In addition to endoscopic polypectomy, which has been shown to reduce the incidence of colorectal cancer, epidemiological studies have shown that individuals reporting a regular intake of aspirin and other nonsteroidal anti-inflammatory drugs, such as Celecoxib, a COX2 inhibitor, have a reduced risk of developing colorectal polyps and cancer. Interestingly, the *jagged2* gene has been shown to be affected in intestinal IEC18 ras transformed cells that revert to normal after treatment by Celecoxib, suggesting that Celecoxib could mediate its beneficial effects on colon cancer development through the inactivation of the Notch pathway.[61]

Regarding chemotherapy approaches in colorectal cancers, GSI was reported by Akiyoshi et al to enhance taxane-induced mitotic arrest and apoptosis of colon cancer cells (DLD1 and SW480) in vitro and in vivo, suggesting that GSI could be a new therapeutic modality for overcoming resistance to taxanes in colorectal cancers.[48] Similarly, oxaliplatin, 5-fluorouracil (5-FU), or SN-38 (the active metabolite of irinotecan) induce an increase in the activity and expression of the γ-secretase complex and subsequently Notch-1 activation and enhanced Hes-1 expression. Combined oxaliplatin treatment with GSI prevented the induction of NICD by this chemotherapeutic agent, blunted Hes-1 activation and subsequently sensitizes cells to chemotherapy. This chemosensitization was mediated by Notch-1, as inhibition of Notch-1 with siRNA enhanced chemosensitivity and prevented the induction of pro-survival pathways (mostly phosphoinositide kinase-3/Akt), whereas over-expression of NICD increased chemoresistance. Because Notch-1 receptor, as well as its downstream target Hes-1, is up-regulated with colon cancer progression, colon cancer cells may up-regulate the Notch signaling pathway as a protective mechanism in response to chemotherapy. Therefore, combining GSIs with chemotherapy may represent a novel approach for treating metastatic colon cancers by mitigating the development of chemoresistance.[62]

CONCLUSION

The Notch pathway is a key regulator of many developmental processes during fetal and adult differentiation. Many of the general Notch functions such as stem cell gate keeper, influencing binary cell fate decisions or induction of terminal differentiation processes were first described in invertebrates and subsequently confirmed in self-renewing organ systems of mammals. In the intestinal epithelium, a symmetrical role of Notch signaling pathway exists between homeostatic self-renewal and cancer. Under physiological conditions, activation of Notch occurs in the crypts and is involved in the maintenance of proliferative potential and in absorptive cell lineage specification of intestinal progenitors. In parallel, Notch signaling pathway was demonstrated as constitutively active in colorectal cancer and this might involve deletions and epigenetic silencing of some components of the Notch cascade, such as Hath1. Direct downregulation of Notch ligands, Notch receptors and Notch downstream targets via silencing RNA or via pharmacologic inhibition of γ-secretase has shown therapeutic effects. Overall, inhibition of Notch signaling in CRC is able to suppress the cell growth, to block neovascularisation of the tumor and to enhance sensitivity to cytotoxic chemotherapy. Although additional studies will be needed to better define the oncogenic interplay between Notch and Wnt signaling cascades in the development of colorectal cancers in human, Notch activation is suggested as a crucial mediator of colorectal adenomas initiation. In the emerging context of cancer stem cells derived solid tumors, investigating the role of Notch signaling in adult intestinal stem cells could provide insights into its potential inhibition as a novel therapeutic approach in treatment of CRC. Interestingly, Notch signaling targeting has been proposed as a promising therapeutic approach in other gastro-intestinal tract related cancers or premalignant conditions, such as malignant mesothelioma or Barrett's esophagus and inflammatory bowel diseases.[34,63,64]

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CHAPTER 22

NOTCH SIGNALING AND BRAIN TUMORS

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Abstract: Human brain tumors are a heterogenous group of neoplasms occurring inside the cranium and the central spinal cord. In adults and children, astrocytic glioma and medulloblastoma are the most common subtypes of primary brain tumors. These tumor types are thought to arise from cells in which Notch signaling plays a fundamental role during development. Recent findings have shown that Notch signaling is dysregulated and contributes to the malignant potential of these tumors. Growing evidence point towards an important role for cancer stem cells in the initiation and maintenance of glioma and medulloblastoma. In this chapter we will cover the present findings of Notch signaling in human glioma and medulloblastoma and try to create an overall picture of its relevance in the pathogenesis of these tumors.

INTRODUCTION TO BRAIN TUMORS

Brain tumors are a heterogeneous group of neoplasms and include all tumors located inside the cranium and the central spinal canal. Primary brain tumors (PBT, also referred to as true brain tumors) arise from cells intrinsic to these cavities, while secondary brain tumors are metastases originating from cancers outside the brain.

PBT are primarily of neuroepithelial origin and are, according to the World Health Organization (WHO) classification, distinguished based on their histological appearance and named based on the cell type they most closely resemble. PBT are further graded as low-grade tumors (non-anaplastic, WHO Grades I and II), or high-grade tumors (anaplastic, WHO Grades III and IV) based on five main features: cytological atypia, anaplasia, mitotic activity, microvascular proliferation and necrosis.[1]

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Gliomas are the most common PBT in adults (70% of all brain tumors) with a yearly incidence of approximately 6/100,000 in western countries.[2] They are categorized as derived from glial tissue and comprise astrocytomas, oligodendrogliomas, mixed oligoastrocytomas and ependymomas. Further distinction is made based on their grade of anaplasia and the astrocytic tumors include pilocytic astrocytomas (WHO Grade I), diffuse astrocytomas (WHO Grade II), anaplastic astrocytomas (WHO Grade III) and glioblastoma multiforme (GBM, WHO Grade IV). GBM accounts for 50-60% of all intracranial gliomas[3] and is recognized as the most aggressive PBT in adults with a median survival around 15 months.[4] GBM is preferentially located in the cerebral hemispheres and is diagnosed based on the presence of vascular proliferation and necrosis, which thus are considered as hallmarks of GBM. Primary GBM, which represents the vast majority of glioblastomas (95%), arises rapidly as de novo tumors without evidence of pre-existing lesions and affect mainly elderly people (mean age 62 years).[2] Loss of heterozygosity (LOH) of 10q and mutations in the Phosphatase and Tensin homolog (*PTEN*) gene are often found in primary GBM.[2,5] Likewise are over expression and abnormal activity of the epidermal growth factor receptor (EGFR), as a result of amplification and/or mutation, frequently observed and are associated with a more aggressive disease.[5-7] Secondary GBM progresses slowly from Grade II or III astrocytomas and often harbors inactivating mutations of the tumor suppressor gene *p53* as does diffuse astrocytomas and anaplastic astrocytomas.[2] Treatment of GBM remains a significant therapeutic challenge, as the majority of gliomas are difficult to operate due to their location and invasive nature. Furthermore are radiation- and chemotherapy often ineffective and relapse is almost certain, why GBM remains thus far incurable.

Astrocytomas are also common in children, accounting for approximately half of the PBT cases, while the remaining 50% includes tumors rarely seen in adults.[8] Of the latter, medulloblastoma (MB, WHO Grade IV) is the most common embryonal PBT with an annual incidence estimated to approximately 0.7/100,000 for children under the age of 15. MB arises in the cerebellum and has an early onset, is fast growing, invasive, predominately displays neuronal differentiation and has a tendency to metastasize through the cerebrospinal fluid. Histologically, MB is characterized by densely packed small round cells with hyperchromatic nuclei surrounded by scanty cytoplasm, typically exhibiting high mitotic activity and anaplasia. Angiogenesis and necrosis are however uncommon.[1,9] The most common cytogenetic alteration in MB is isochromosomy 17, i{17}q, which results in trisomy 17q and monosomy 17p.[10] Together with 6q gain and amplification of *MYC* and *MYCN*, additional common genetic modifications, this has been associated with poor survival.[9-12] Furthermore, aberrant activation of the Wnt and Hedgehog pathways has been implicated in the pathogenesis of a subset of MB.[13] WHO has further classified four different MB histological subtypes, which have been shown to be associated to clinical behavior. As such, desmoplastic/nodular MB and MB with extensive nodularity correlate with an average-risk disease, whereas anaplastic MB and large cell MB tends to display a more aggressive clinical course and are characterized as high-risk disease.[12,14,15] Current multimodal treatment of MB has led to a five-year disease-free survival of nearly 90% for children with average-risk and a 60-65% survival rate for children with high-risk disease.[15] However, survivors have a highly elevated risk for increased morbidity and mortality later on in life, with disease relapse being the single most common cause of death. They moreover have an increased susceptibility for developing long-term complications as a consequence of neurological effects caused by the tumor or treatment.[15-17]

Astrocytic gliomas and medulloblastomas represent the most common PBT in adults and children and are believed to originate from immature cells types in the brain[18-21] in which Notch signaling plays a fundamental role during normal development.[22] As such, we will in the following focus on Notch signaling in these tumor types.

EXPRESSION OF NOTCH PATHWAY COMPONENTS AND GLIOMA GRADE

Several studies have reported abnormal expression of various components of the Notch signaling cascade in human gliomas. The Notch pathway is involved in the normal development of the brain and in the maintenance of neural progenitor cells,[22] and thus plays a key role in tissues from which gliomas are thought to arise. However, the cell(s) of origin for gliomas is yet to be identified and it is possible that gliomas of different grades might arise from cells displaying various levels of differentiation (Fig. 1). As such, they could also differ in their expression of the Notch pathway components. Indeed, Hash-1 and Delta-like ligand (Dll)-1 expression has been correlated to Grade II- and III- gliomas and secondary GBM,[23,24] and Hash-1 expression has been suggested to distinguish progressive gliomas and secondary GBM from primary GBM.[23] Furthermore, expression of Notch-1, -3 and -4 has been associated with increasing tumor grade in astrocytomas,[25,26] and expression of the downstream Notch target Hey-1 has been shown to correlate with glioma grade, with the highest expression in GBM, although there is no distinction between primary and secondary GBM in this study.[27] As such, it could be argued that low-grade gliomas and progression into secondary GBM, are characterized by inactive Notch signaling, whereas primary GBM arising de novo is characterized by an activated Notch cascade, reflecting a more immature and aggressive phenotype.[23,28]

FUNCTIONAL SIGNIFICANCE OF NOTCH SIGNALING IN GLIOMAS

Emerging evidence indicate a functional role for Notch signaling in gliomas. Inhibition of Notch-1 has been shown to increase cell death, decrease proliferation and induce cell cycle arrest in glioma cell lines, whereas constitutive activation of Notch-1, as obtained by exogenous expression of intracellular Notch-1 (ICN-1), leads to increased proliferation.[26,29-33] Furthermore, Hey-1 expression, indicative of activated Notch signaling, has been shown to be crucial for proliferation of glioma cell lines expressing high endogenous levels of Hey-1, whereas it is non-essential in low Hey-1 expressing cells.[27] These results indicate that an active Notch cascade is involved in maintaining glioma cells in a proliferative state, which is further strengthened by the notion that inhibition of Notch-1 in vitro leads to changes in morphology and expression of various differentiation markers, indicating cellular differentiation.[29,30] Inhibition of Notch-1 or Dll-1 in vivo leads to delayed tumor growth and longer survival,[26,29] and expression of Hey-1 has been linked to dismal prognosis in GBM patients.[27,34] In addition, Tenacin-C, which has been correlated to increased malignancy and poor clinical outcome in gliomas and which has furthermore been shown to promote glioma cell migration, has recently been identified as a direct Notch target.[35] In summary, these results point towards a role for active Notch signaling in maintaining glioma cells in

an aggressive, immature and proliferative state and correlates well with an activated Notch cascade in high-grade gliomas.

NOTCH SIGNALING IN MEDULLOBLASTOMA—ONCOGENE VERSUS TUMOR SUPPRESSOR

The output of Notch signaling in cancer can be either oncogenic or tumor suppressive, most likely as a consequence of the normal role of Notch signaling in the particular cell type giving rise to the tumor.[36,37] In brain tumors, this is perhaps best exemplified in neuronal. These embryonic tumors are thought to arise from cerebellar cortex granule neuronal precursor (GNP) cells that differentiate in the external granule layer (EGL) where most MB occur. In GNPs, Notch-2 is expressed and is required for maintaining a proliferative and undifferentiated phenotype.[38] In line with this, Notch-2 is up regulated in MB and promotes cellular proliferation upon overexpression.[25,39] On the contrary, Notch-1 is seldom expressed in MB and when overexpressed it leads to growth inhibition in MB cell lines.[25,39] As such, Notch-2 is believed to function as an oncogene in medulloblastoma whereas Notch-1 is thought to function as a tumor suppressor.[39] Even though Hes-1 is considered to be a target of canonical Notch signaling and thus would be activated by both Notch-1 and Notch-2 receptors, it appears as if Notch-2 is the major activator of Hes-1 expression in MB as Hes-1 expression correlates to Notch-2.[39] In addition, it seems as if Hes-1 is a key player in mediating the oncogenic effects of Notch-2 signaling in MB as its expression correlates significantly to shorter patient survival.[39] Furthermore, inactivation of Hes-1 through the microRNA-199b-5p leads to increased differentiation and decreased proliferation in MB cells, again highlighting the role of an active Notch cascade, through Hes-1, in maintaining the tumorigenic potential of MB.[40]

BRAIN CANCER STEM CELLS IN GLIOMAS AND MEDULLOBLASTOMAS

With the identification of a minority population of stem cell-like cells within the heterogeneous cell mass that comprise brain tumors, brain cancer is increasingly being viewed as a stem cell disease. This sub-population of cells is termed brain cancer stem cells (bCSC), or brain tumor initiating cells, as they show pronounced similarities to normal neural stem cells (NSC) and have tumorigenic potential. bCSC are believed to play a significant role in tumor initiation, progression, treatment resistance and relapse,[18,41-46] and are as such a promising new target in the treatment of PBT.

The neurosphere culture system and analysis introduced in the early 1990s to identify NSC[47] has permitted in vitro identification and characterization of bCSC in a variety of PBT including GBM and MB.[48-55] When grown in this culture system, which is based on a serum-free environment with specific growth factors added, NSC and bCSC form floating aggregates called neurospheres. These represent clonal, single cell-derived, clusters of proliferating cells and can be sub-cultured to generate secondary neurospheres, which are used to characterize their self-renewing potential.[52,56,57] Neurosphere-forming cells are further characterized by the expression of stem cell markers such as Nestin and CD133.[33,46,48,52,58-61] By adding serum or withdrawing growth factors, both NSC and bCSC differentiate into cells of the three neural lineages (astrocytes, oligodendrocytes and neurons), hence they are multipotent,[48,50,52,60,62] although the differentiation pattern

might be somewhat disturbed for bCSC.[63,64] In addition to displaying NSC characteristics, bCSC are tumorigenic and when transplanted into immunocompromized mice, secondary xenograft tumors are formed with phenotypic and cytogenetic similarities to the patient tumor from which they were originally derived.[49,51-53]

EXPRESSION OF NOTCH PATHWAY COMPONENTS IN bCSC

It is yet to be established whether bCSC arise from NSC or from dedifferentiation of more mature CNS cells, or even from the tumor cells themselves and as such are a result of tumor progression instead of the initiator. Human PBT are often located in association to neurogenic areas in the brain,[20,21,65,66] such as the subventricular zone (SVZ) lining the lateral ventricles and in the dentate gyrus of the hippocampus,[58] where Notch signaling plays a key role in NSC maintenance and cell fate decisions.[22,37,67-69] Therefore, it is no surprise that the Notch receptors and ligands and several key mediators of the Notch signaling cascade have been found aberrantly expressed in bCSC isolated from various grades and types of human PBT,[19,48,51,60,70] indicating a functional role for Notch signaling in bCSC (Fig. 1).

GBM derived neurosphere cultures more closely mimic the pheno- and genotype of the original patient tumor than GBM cell lines established the traditional way, in the presence of serum.[51,71] Interestingly, when compared in a global gene expression analysis, GBM derived neurosphere cells cluster together with normal NCS and, moreover, express high levels of genes involved in CNS function and development as well as NSC associated genes, such as *Notch-1* and *-4* and *Dll-1* and *-3*.[51] Neurosphere cultures are usually divided into three groups based on their growth characteristics and categorized as spherically-, semi-adherently- or adherently growing cultures. The degree of adherent cells is likely to be associated to the degree of differentiated cells. At least in the case of GBM, the three categories can be further distinguished by their level of stemness and tumorigenicity. Cultures that grow exclusively as spheres have a full multipotent potential, express CD133 and display high invasiveness, whereas semi-adherent and adherent cultures have restricted differentiation potential, little or no CD133 expression and are less tumorigenic.[60] Intriguing, the non-adherent sphere cultures show over expression of components belonging to the Notch cascade (Notch-1, Dll-3 and Hes-1) as well over expression of Nestin,[60,72] which also has been shown to be a direct transcriptional target of Notch signaling.[73,74] However, neither semi-adherent nor adherent cultures display increased expression of these genes.[60] In line with this, it has been shown that a high in vitro Notch-1 pathway activity results in a higher frequency of sphere formation from NSC than when Notch-1 activity is low.[75] Cells derived from astrocytoma Grade III are likewise capable of neurosphere formation in serum-free culture conditions and express similar levels of Nestin and the Notch ligand Jagged-1 as GBM neurospheres.[48] When compared to normal brain derived neurospheres neither Grade III nor Grade IV glioma cultures express detectable levels of the Notch ligand Delta*. However, when transferred to serum-containing media and allowed to adhere, thereby inducing differentiation, they gain Delta expression.[48] Dll expression is seen in cells committed to the neuronal lineage,[76] in line with the concept of lateral inhibition[22,77-79] and can in this case thus be associated with a more differentiated phenotype.

*It is not specified which Delta variant that is referred to.

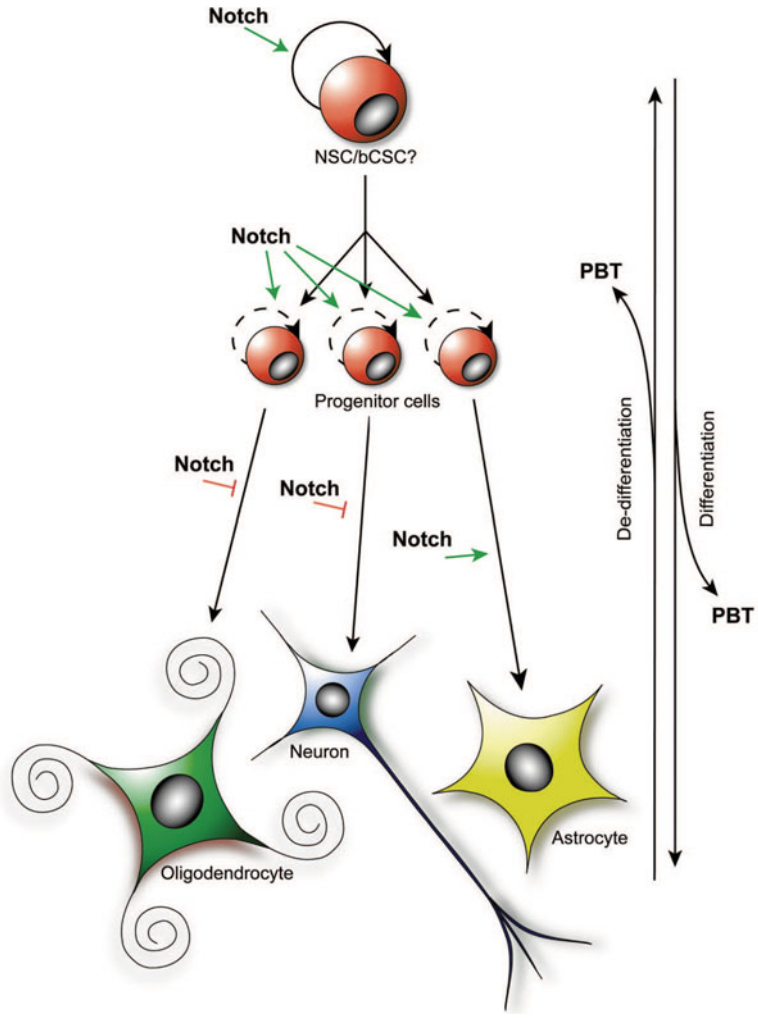


Figure 1. A simplified view of Notch function in NSC and development of brain tumors. Notch signaling is important for NSC and possible bCSC self-renewal. From the stem cells, progenitor cells arise which will generate more and more lineage restricted progenitors. Notch signaling is involved in progenitor self-renewal in some of these developmental steps. Finally, an active Notch cascade is central for the terminal differentiation of astrocytes and on the contrary inhibits the maturation of neurons and oligodendrocytes. The cell of origin for PBT is currently not known. However, it is possible that PBT might arise from transforming events in every developmental step from the NSC to mature CNS cells, in which Notch signaling plays a fundamental role. Modified from: Stockhausen et al. The functional role of Notch signaling in human gliomas. Neuro Oncol 2010; 12(2):199-211, ©2010 with permission of Oxford University Press.

Based on the above outlined observations, it is tempting to speculate that increased differentiation due to low Notch activity decreases tumorigenicity by reducing the bCSC pool and vice versa.

FUNCTIONAL ROLE OF NOTCH SIGNALING IN bCSC

We are only beginning to understand the role of Notch signaling in bCSC, though the number of functional studies addressing this subject is increasing. By transfecting a GBM cell line with ICN-1, a cell line with significantly increased viability and colony- and neurosphere-forming capacity can be obtained when compared to the parental cell line and control cell line transfected with an empty vector.[33] These results are in line with observations showing that the level of intracellular ICN-1 and thus the level of Notch activity, corresponds to the neurosphere forming capacity of Id4 (inhibitor of differentiation 4) transformed murine astrocytes with GBM characteristics.[80] Furthermore, transfection with ICN-2 leads to increased cell growth of GBM derived neurosphere cells,[81] and enlarged fraction of MB cell line derived CD133-positive cells.[70] Supporting a growth- and neurosphere promoting effect of ICN-1 and -2, GBM derived neurosphere cultures exhibit sensitivity to gamma-secretase inhibitors (GSI) as displayed by decreased cell viability, loss of clonogenic growth capacity in soft-agar and reduced neurosphere formation. GSI treatment moreover down regulates Nestin and CD133 expression as well as reduces the CD133-positive cell population in GBM derived neurosphere cells.[19,72,81,82] MB cell lines are likewise responsive to GSI treatment as demonstrated by reduced proliferation and colony formation in soft-agar, increased neuronal differentiation and diminished CD133-positive and Nestin-positive cell populations.[70] The growth of normal human astrocytes is, however, not affected[81] indicating that GSI selectively targets bCSC.

As described above, bCSC have the potential to form xenograft tumors when transplanted into immunocompromized mice. However, when GBM neurosphere cells pretreated with GSI in vitro are injected subcutaneously, no tumor formation is observed when compared to control mice, although small lesions develop at half of the injection sites. Conversely, when neurosphere cells are injected intracranially into nude mice, both GSI treated and control cells are able to form orthotopic tumors, although mice injected with GSI treated cells have a significantly longer survival. Histologically there are no differences between the pretreated and the control tumors.[81] These divergent observations suggest that when growing orthotopically, the highly vascularized micro-environment in the brain is able to counteract the effect of Notch inhibition to promote tumor growth. In opposite, subcutaneous tumors are vascular-poor and as such the proper tumor promoting micro-environment might be absent. This is intriguing as bCSC are abundant in vascular niches,[83,84] were endothelial cells promote bCSC survival and self-renewal and bCSC have further been associated with secretion of the pro-angiogenic factor vascular endothelial growth factor (VEGF) leading to tumor angiogenesis,[42] a crucial step in tumorigenesis. When locally treating intracranially implanted GBM neurosphere cells with GSI, no detectable tumor formation is observed.[81] However, when transfected with ICN-2 GBM neurosphere cells form large intracranial tumors, which show resistance to GSI treatment. In concordance, mice injected with nontransfected cells has a significantly prolonged survival compared to mice injected with ICN-2 transfected cells, upon local GSI treatment.[81] When GSI treated MB cell lines are injected subcutaneously, only small lesions are formed at the injection site as in the case of GBM neurosphere cells.[70] Furthermore, 75% of the mice injected subcutaneously with MB cells fail to form tumors upon systematic treatment with GSI, when compared to control treated mice and mice injected with ICN-2 transfected cells.[70]

Taken together, it can be speculated that an active Notch pathway is important for maintaining the stem cell characteristics and tumorigenic potential of bCSC in GBM and

MB and that bCSC and tumor formation can be targeted through the Notch signaling pathway.

NOTCH SIGNALING AND INTRACELLULAR CROSS-TALK IN GLIOMAS

As mentioned above, primary GBM tumors are often associated with amplification and/or overexpression of EGFR.[85-87] As such it is intriguing that there are several reports on cross-talk between the Notch- and EGFR signaling pathways in cancer.[88-92] EGFR is expressed in neurogenic regions of the brain, such as the SVZ,[93] and Notch signaling has been suggested to regulate survival and proliferation of NSC through interaction with pathways downstream of EGFR.[94] Two of the main downstream pathways of EGFR are the RAS/MEK/ERK- and the PI3-K/AKT pathways, which are known to be involved in cellular processes such as proliferation, migration and resistance to apoptosis. In human RAS-transformed astrocytes, Notch-1 expression is up regulated and upon inhibition of Notch signaling, proliferation and viability is decreased along with induced differentiation.[30] Thus, it seems as if Notch signaling is involved in maintaining RAS-transformed astrocytes in an undifferentiated, proliferative state. Although, as constitutively activated Notch-1 fails to transform immortalized astrocytes or enhance tumorigenicity of RAS-transformed astrocytes on its own it is still not known whether Notch signaling is involved in the transformation process itself or rather is a result of it.[30] However, combined expression of activated Notch-1 and K-RAS in glial progenitors induces periventricular stem cell-like lesions in mouse SVZ, indicating that Notch-1, in synergy with RAS, indeed plays a role in glial transformation.[74] The discrepancy regarding the importance of Notch signaling in transformation of astrocytes versus glial progenitors could refer to differences in differentiation grade between the two cell types. As such, it is possible that Notch signaling plays a greater role in transformation of less differentiated cells and would point towards a role for Notch signaling in gliomas arising from NSC, possibly resulting in high-grade gliomas.

Apart from Notch-RAS interactions, phosphorylation and thus activation, of AKT is linked to Notch signaling in gliomas (Fig. 2). Constitutively activated Notch-1 leads to phosphorylation of AKT and increased glioma growth in vitro, whereas inhibition of Notch-1 suppresses AKT activation along with decreased glioma viability both in vitro and in vivo.[26,31,32] Furthermore, in glioma neurosphere cell lines, Notch pathway inhibition leads to blocked AKT phosphorylation concomitant with decreased proliferation of stem-like GBM cells.[81] As Notch signaling has been suggested to promote survival of NSC through AKT, it is also possible that Notch is involved in regulating GBM bCSC through the same process.[94]

Thus, Notch signaling in gliomas can be coupled to both the RAS/MEK/ERK- and the PI3-K/AKT pathways. Although, it is important to note, that their activity is not necessarily a consequence of EGFR signaling as both these pathways can be activated through a plethora of other growth factor receptors. However, when dividing glioma tumor specimens into subclasses depending on protein expression, the Notch pathway is activated among tumors associated with EGFR signaling, in most cases as a consequence of *EGFR* amplification or mutation.[95] This correlation can only be found at the protein level and indicates that posttranscriptional mechanisms are involved in regulation of the Notch pathway.[95] Indeed, enhanced translation of Notch-1 mRNA is induced by RAS and AKT, resulting in increased Notch-1 protein expression, in a glioma mouse model.[96] In contrast to the correlation at the protein level between the Notch pathway and EGFR in

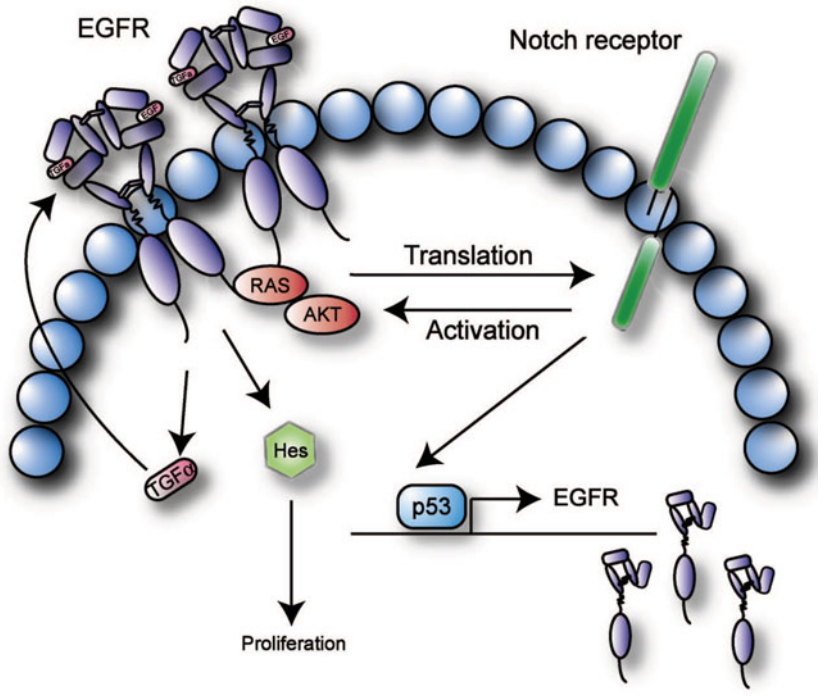


Figure 2. Schematic overview over the cross-talk between Notch and EGFR signaling in gliomas. See text for details. Modified from: Stockhausen et al. The functional role of Notch signaling in human gliomas. Neuro Oncol 2010; 12(2):199-211, ©2010 with permission of Oxford University Press.

gliomas amplified for the *EGFR* gene, Notch-1 and EGFR mRNA correlates in high-grade gliomas lacking *EGFR* amplification.[97] When inhibiting Notch-1 in glioma cell lines, EGFR mRNA and protein is down regulated whereas forced expression of Notch-1 leads to up regulation of EGFR expression in a p53 dependent manner.[26,97] These results indicate that in non-*EGFR* amplified gliomas, with wild-type p53, EGFR expression is under the transcriptional control of Notch signaling, whereas in tumors amplified for *EGFR*, Notch pathway components are up regulated at the translational level, possibly through increased activation of the EGFR downstream pathways RAS/MEK/ERK and PI3-K/AKT. As the majority of *EGFR* amplified gliomas belong to the group of primary GBM that also in most cases are wild-type for p53, increased Notch levels, as a result of EGFR driven translation, could lead to an increase in EGFR transcription through Notch and p53, thus creating a positive feedback loop between EGFR and Notch signaling (Fig. 2). EGFR is known to drive the expression of its own ligand TGF-α, which has also been correlated to increased tumor grade among gliomas.[98,99] In addition, TGF-α has been shown to induce expression of Hes-1, as well as promoting Hes-1 nuclear translocation in glioma cells, resulting in increased proliferation (Fig. 2).[100] This further highlights that the cross-talk between Notch and EGFR signaling might be of importance for the malignant character of primary GBM. However, this is thus far purely speculative and as such the relevance of the cross-talk between Notch and EGFR in glioma needs further clarification.

NOTCH SIGNALING CROSS-TALK IN MEDULLOBLASTOMA

Apart from Notch signaling, a number of developmental pathways have been linked to MB. One of these is the Hedgehog (HH) signaling pathway, implicated in maintenance of stem cells in several tissues including the nervous system.[101] There is now emerging evidence that this pathway also co-operates with Notch signaling in the onset and maintenance of MB. When expressing an active form of Smoothened, a key component of the HH pathway, in mouse cerebellar cortex GNP cells, MB are formed, along with up regulation of the HH target and transcriptional regulator, Gli. In addition, Notch-2 and Hes-5 are up regulated and when inhibiting Notch and HH signaling simultaneously in MB cell lines, cell viability is more impaired as compared to inhibition of either pathway alone.[102] Furthermore, in MB from mice heterozygous for the HH negative regulator Patched, HH signaling is activated concomitant with upregulation of Notch-2, Hes-1 and -5, RBP-jκ and Jagged-1.[103] As such, when modulating HH signaling there is an effect on the Notch pathway. However, interaction between the two pathways is reciprocal and as such, components of the Notch signaling pathway are also involved in regulating HH signaling. The tumor suppressor Numb, which is involved in repression of Notch signaling through receptor degradation, is down regulated in MB. One possible explanation for this is the presence of Musashi in MB, an inhibitor of Numb and thus activator of Notch signaling.[104] Apart from regulating Notch signaling, Numb expression also promotes degradation of Gli, which results in inhibition of HH signaling, leading to blocked proliferation and induced differentiation of MB cells.[105]

HYPOXIA AND ANGIOGENESIS—MEDIATORS OF NOTCH SIGNALING IN GLIOMAS

Inadequate oxygenation, as a result of malformed and leaky tumor vessels, leads to hypoxia and necrosis, which are hallmarks of GBM. At hypoxia, the hypoxia inducible factors (HIF)-1α and –2α are stabilized and initiate the transcription of pro-angiogenic factors such as VEGF, involved in the formation of new blood vessels. Even if hypoxia elicits an angiogenic response, the resulting tumor vessels are often dysfunctional and hypoxic areas will remain. In GBM, chemically induced hypoxia augments the expression of ICN-1 as well as the downstream targets Hes-1 and -5 and the ligand Jagged-1.[106] Moreover, in primary GBM cell cultures grown at hypoxia, mastermind-like 3 (MAML3), which is involved in mediating the transcriptional output of Notch signaling, is up regulated in a Hif-2α dependent manner.[28] Furthermore, MAML3 is significantly up regulated in primary GBM as compared to secondary GBM, correlating with increased CD133 expression and thus a larger fraction of bCSC in these tumors.[28] These results, are in line with the role of Notch in NSC at hypoxia, where Notch signaling is required to maintain the cells in an undifferentiated state.[107]

The Notch ligand Dll-4 is crucial for proper formation of blood vessels during embryogenesis and increasing evidence indicates a role for Dll-4 also in tumor angiogenesis.[108-110] Blocking of Dll-4 in vivo leads to increased tumor vessel density, however the vessels are poorly functional and as a result tumor growth is inhibited.[111,112] Dll-4 mRNA has been shown to be expressed in glioma endothelial cells and furthermore, in some cases also in the tumor cells themselves.[111,113] When expressing Dll-4 in a glioma xenograft model, tumor growth is enhanced.[113] Although vessel number and density are

decreased, tumor perfusion is enhanced leading to reduced intratumoral hypoxia and necrotic areas.[113] In addition, mRNA for both Dll-4 and Hes-1 is up regulated in host endothelial cells in response to glioma Dll-4 expression.[111,113] This indicates that Dll-4 expressed in glioma cells can activate Notch signaling in nearby endothelial cells, leading to neo-vascularization as has been shown earlier with other Notch ligands in head and neck squamous carcinoma cells.[92] The postulation that Notch signaling plays a role in glioma angiogenesis is further corroborated by decreased tumor vascularization upon treatment with GSI in a glioma xenograft model,[114] however the exact mechanism of how Notch signaling affects glioma angiogenesis is still not known. Although, it is tempting to speculate that Dll-4 is a key player as its expression can be regulated both by Hif-1α and VEGF that in turn are expressed in response to hypoxia but also in response to TGFα/EGFR signaling, features of high-grade gliomas such as primary GBM.[115,116] Additionally, it is considered that the bCSC fraction is larger in high-grade gliomas than in lower grades,[117,118] and as this cell population has been accounted for production of VEGF,[42] there is a further link between bCSC and Dll-4 and thus Notch signaling, in glioma angiogenesis.

NOTCH AS A THERAPEUTIC TARGET IN BRAIN TUMORS

Due to its role in tumorigenesis the Notch signaling cascade is increasingly considered as a target for cancer therapy. The most widespread approach to inhibit Notch signaling is by using GSI, which is also currently planned in a Phase II clinical trial for the treatment of patients with recurrent or progressive GBM†. However, it is important to emphasize that GSIs not only target the Notch receptors and furthermore are not tumor specific, thus unwanted side effects are most likely to occur. One documented side effect of GSI is metaplasia of goblet cells in the small intestine.[119] However, by administrating the drug intermittently, this effect is alleviated, probably by allowing proper differentiation of intestinal stem cells. On the other hand, the notion that Notch inhibition affects stem cells could also be an advantage, as this could indicate that bCSC are sensitive to Notch inhibition. Indeed, GSI enhance cell death and reduce growth of glioma bCSC and furthermore sensitize them to radiation.[120] This is an important notion as bCSC have been shown to mediate radiation resistance in gliomas.[43] In response to radiation, bCSC have been shown to activate DNA damage checkpoint response and increase DNA damage repair activity, thus decreasing apoptosis.[43] Moreover, GBM neurosphere cell cultures and xenografts are enriched in CD133-positive cells after radiation[43] and recurrent GBM harbor more CD133-positive cells as compared to newly diagnosed GBM tumors.[44] It could thus be argued that present glioma treatment, fails because it only kills the bulk of the tumor, whereas the tumor initiating bCSC escape and are able to regenerate the tumor and cause relapse. Upon radiation, Notch-responsive promoter activity is induced and Notch target genes are up regulated in glioma derived CD133-positive cells in vitro.[120] GSI treatment combined with radiation therapy increase radiation induced cell death when compared to either radiation or GSI treatment alone and significantly inhibits neurosphere formation. Transfecting the cells with either ICN-1 or -2 attenuates the additive effect of radiation and GSI, whereas Notch-1 or -2 knock down by short-hairpin (sh) RNA sensitizes CD133-positive cells to radiation while the CD133-negative cells are irresponsive to GSI treatment regardless of radiation.[120] These data suggest that Notch

†http://www.clinicaltrials.gov

activity protects bCSC from radiation induced cell death and that blocking this pathway renders bCSC more sensitive to radiation.

Anti-angiogenic treatment using the VEGF inhibitor bevacizumab (Avastin®) is currently one of the best approaches to treat GBM.[121,122] However, even though this regimen has had great success, the tumors invariably relapse. This is thought to be a result of tumor adaptation by activation of alternative pro-angiogenic pathways.[123] As Notch signaling, through the expression of Dll-4 is involved in tumor angiogenesis, activation of this pathway might be one way for the tumors to circumvent bevacizumab treatment. This is exemplified in an in vivo study where glioma xenografts expressing Dll-4 continued to grow at the same rate as control-treated tumors after termination of bevacizumab administration, although they were initially sensitive to the treatment.[113] When inhibiting Notch signaling by soluble Dll-4 both bevacizumab-sensitive and -insensitive tumors were affected, indicating that targeting Notch signaling, in addition to VEGF, would be a promising approach in the therapy of high-grade gliomas.

CONCLUSION

Notch signaling has a fundamental function in the cells from which human brain tumors are thought to arise. This is most likely superimposed on its role in the formation of these tumors and increased knowledge of physiologic Notch signaling might lead us in the direction of solving its role in brain tumorigenesis. Human brain tumors such as high-grade gliomas and medulloblastomas are characterized by the presence of bCSC, increased growth factor signaling, hypoxia and angiogenesis, processes in which Notch signaling could be a key player. In addition, as all of these features interact in a complex pattern, it could be speculated that targeting several of these pathways in concert could lead to increased tumor control.

There are strong indications that bCSC might by the cell responsible for tumor formation and treatment failure in PBT and there are several lines of evidence indicating a functional role for Notch signaling in bCSC maintenance and tumor initiation and aggressiveness. As such, aiming at the Notch pathway could result in inhibiting the brain tumor initiating cell population, the bCSC and thereby represent a novel therapeutic target.

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CHAPTER 23

NOTCH INHIBITION AS A PROMISING NEW APPROACH TO CANCER THERAPY

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Abstract: The Notch pathway powerfully influences stem cell maintenance, development and cell fate and is increasingly recognized for the key roles it plays in cancer. Notch promotes cell survival, angiogenesis and treatment resistance in numerous cancers, making it a promising target for cancer therapy. It also crosstalks with other critical oncogenes, providing a means to affect numerous signaling pathways with one intervention. While the gamma-secretase inhibitors are the only form of Notch inhibitors in clinical trials, other forms of Notch inhibition have been developed or are theoretically feasible. In this chapter we review the rationales for Notch inhibition in cancer and then discuss in detail the various modalities for Notch inhibition, both current and speculative.

INTRODUCTION: BACKGROUND OF THE NOTCH PATHWAY IN CANCER

In the current era in oncology, much of the hope for powerful new therapies lies with targeted inhibition of pathways dysregulated in cancer. An initial wave of targeted pathway inhibitors has yielded some successes but more disappointments and major efforts are underway to refine our application of some of these approaches. However, there is no slowdown in attempting to find newer and perhaps more effective targets in cancer cells and the Notch pathway is generating growing enthusiasm in this regard. As is described in detail elsewhere in this volume, Notch is a key player in development, stem cell maintenance and cell survival and its specific roles in individual cancers are covered in other chapters here. In this chapter, the rationale for Notch inhibition as a

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cancer therapy and its potential drawbacks will be discussed, with extended description of established and experimental methods for Notch inhibition.

RATIONALE FOR NOTCH INHIBITION

Numerous functions have been ascribed to Notch, with some of these helping to explain its cancer-promoting effects in many tissues. Notch helps maintain certain stem cell populations,[1-5] but interestingly it is also a master regulator of cell fate at critical differentiation branch points in various organ systems.[5-8] Notch seems more likely to play an oncogenic role in cell types that it favors in development and differentiation, such as glial cells or T-cells.[9-12] Notch activity promotes cell survival and has anti-apoptotic function[13-15] and numerous mechanisms have been proposed for this. It can also drive cell division in some settings and in some settings may be required for the cell cycle.[16,17]

Notch is one of the most powerful of the stem cell-promoting pathways, in conjunction with the Hedgehog and Wnt pathways, making it highly relevant for cancer given the undifferentiated/de-differentiated state of most cancer cells. Stem cell pathways such as Notch may be especially attractive targets given the growing evidence for the cancer stem cell hypothesis. This hypothesis states that cancers contain a usually small subpopulation that retains stem cell character and gives rise to the other cells making up tumors (reviewed in refs. 18,19). Various terms exist for this subpopulation, including "cancer-initiating cells," "cancer stem cells," or, given the uncertainty about their nature, "cancer stem-like cells." Despite variability in nomenclature, there is general agreement on the criteria that define these cells in the laboratory. Their isolation and culture has allowed detailed study of cancer stem cells and a number of features have emerged. They are capable of unlimited self-renewal, generation of more differentiated progeny and formation of cancers in animal models.[20,21] These cells are more resistant than bulk cancer cells or established older cancer cell lines to standard treatments such as chemotherapy and radiation.[22,23] However, cancer stem cells seem equally sensitive—or even more so—to potential therapies blocking prominent stem cell pathways like Notch.[24-26] Inhibition of these pathways may cause differentiating effects in cancer stem cells, as well as more commonly seen cytotoxic effects. In keeping with this, a few reports have shown differentiating effects in cancer stem cells secondary to Notch inhibition.[24,26]

Some of the impact of Notch inhibition in cancer cells results from its extensive crosstalk with critical cancer proteins and pathways. Numerous studies have shown that Notch activity sustains the PI3kinase/Akt pathway[27-30] and Notch has also been demonstrated to operate in an interdependent fashion with the Ras pathway.[31,32] Notch regulates expression of important receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor receptor-1 (VEGFR-1)[33-35] and also interacts with fibroblast growth factor receptor (FGFR) signaling.[36] Notch and the NF-kB pathway are intimately intertwined, with multiple points of interaction described[37-41] The myc oncogene is a direct target of Notch, mediating much of the oncogenic effects of Notch in T-cell malignancies.[42] In some instances, other oncogenic pathways have been shown to boost Notch or its downstream activity, as is the case for the hypoxia/HIF-1alpha pathway.[43] Most of the best-known oncogenic pathways have been shown to cross-talk with the Notch pathway at some level; however, it is important to note that some of these interactions are context-dependent and do not occur in all cellular backgrounds.

The direct effects of Notch inhibition on cancer cells may vary. Given the interaction of Notch with important anti-apoptotic pathways such as Akt, it is perhaps not surprising that Notch inhibition has most frequently been shown to trigger apoptosis in cancer cells.[14,15,24,29,33,44] Notch inhibition has also been shown to slow cancer cell proliferation, though Notch activity has generally not been considered essential for the cell cycle. However, some evidence indicates that there may be important roles for Notch in the cell cycle in some settings.[45] Senescence has also been linked to the Notch pathway. The downstream mediator of Notch HES1 has been shown to play a critical role in blocking senescence,[46] and this is supported by recent results presented at a meeting that the combination of a Notch-inhibiting agent and a chemotherapy drug triggers senescence in glioblastoma cells.[47] While Notch inhibition has not yet been associated with autophagy in cancer cells, this may just be a matter of time given the connections of Notch to Akt/mTOR signaling.

While Notch blockade can have direct inhibitory effects on cancer cells, it also may influence cancer indirectly through impacting cancer-supporting processes such as angiogenesis. A number of reports have shown direct antiangiogenic effects from Notch inhibition.[48-51] A major role for Notch in blood vessels is supported by the vascular nature of the defects in the human disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), caused by NOTCH3 mutations.[52] Furthermore, in mice the knockouts of NOTCH1 or its ligands Delta-like-1 or Jagged-1 are embryonic lethal due principally to vascular defects.[53,54] Recently, signaling via the Notch ligand Delta-like-4 was found by multiple groups to regulate endothelial sprouting and its inhibition led to disordered and unproductive endothelial growth and decreased tumor size—even in cancers resistant to VEGF inhibition.[49,51,55] In addition, small-molecule Notch-inhibiting drugs have been shown to have potent antiangiogenic effects in animal models of cancer.[56-58] The precise mechanisms by which Notch regulates the vasculature seem to be diverse. Delta-like-4/Notch signaling directly regulates angiogenic endothelial cells, and Notch also seems to regulate aspects of vascular development such as arterial versus venous fate.[59-61] More specifically for cancer, endothelial cells and cancer cells have been shown to communicate via Notch signaling to promote angiogenesis and cancer growth.[62] Notch also regulates expression of vascular endothelial growth factor recptor-1 (VEGFR-1), a key receptor for vascular formation.[35] The diverse roles of Notch in angiogenesis may have implications for cancer; by blocking processes such as angiogenesis, Notch inhibition may be valuable even in cancers whose cells are not directly sensitive.

As is becoming clear for many targeted inhibitors in cancer, Notch inhibition may be best not as solitary therapy but in combination with other agents. A growing body of evidence demonstrates that Notch inhibitors sensitize to more standard treatments such as radiation therapy and chemotherapy.[47,63,64] Notch inhibitors are also being assessed in combination with other targeted inhibitors,[65] and such an approach may be important to maximize effects given the likelihood that most cancers will have lesions in multiple oncogenic pathways. That single cancers are driven by genetic lesions in multiple pathways has been extensively demonstrated in glioblastoma, the most common and aggressive primary brain tumor.[66] This has raised concerns that inhibition of individual signaling pathways will almost never be sufficient for cancer therapy, despite the phenomenon of "oncogene addiction" established in recent years. Notch inhibition may be particularly effective when combined with inhibitors of other key stem cell pathways. For example, recently-presented but still unpublished results show

potent anti-cancer effects from combining a Notch-inhibiting drug and a Hedgehog pathway inhibitor in glioblastoma stem cell lines.[67]

POTENTIAL RISKS OF NOTCH INHIBITION

Notch inhibition as cancer therapy may also pose significant risks, and the potential risks and benefits are summarized in Table 1. One major side effect that emerged from the pioneering trials of a first-line Notch-inhibiting drug is gastrointestinal toxicity and diarrhea.[68] This is likely an on-target toxicity, given reports that Notch drives gastrointestinal precursor cells toward an epithelial fate and away from a secretory cell fate; Notch inhibition thus causes an imbalance with too many secretory goblet cells.[5,69] This showed its potential to be a dose-limiting toxicity in these earliest trials, and it is likely one that will be problematic for any systemically-delivered Notch inhibitor. However, potential answers to this have already arisen. It has been found that intermittent dosing schedules of a Notch inhibitor can largely spare the gut while maintaining anti-tumor efficacy. In addition, it has been found that corticosteroid administration, already a component of some cancer regimens, may help ameliorate the gut toxicity of Notch inhibition.[70]

Two other theoretical risks of long-term Notch inhibition have been posited. One is the potential for damage to normal stem cells in the body, which may rely on Notch signaling to varying degrees. Possible results of this are difficult to determine, but could include anything from hematopoietic collapse to subtle cognitive decline. No signs of such toxicities have been uncovered in the earliest clinical trials, but the dosing was relatively short in those trials. Even if such toxicities emerge, it is possible that they too might be addressed by intermittent dosing of a Notch inhibitor. The other theoretical risk may be even more concerning, as it involves an increased incidence of certain cancers. While Notch plays an oncogenic role in most tissues, it acts as a tumor suppressor in some, such as certain skin cells, neuroendocrine lung cells and B-cells.[71-73] Thus long-term Notch inhibition may increase the risk of cancers in these cellular compartments, though this

Table 1. Potential advantages and disadvantages of Notch inhibition for cancer therapy

Advantages	Disadvantages
May be especially active against resistant tumor stem cell subpopulation	Gastrointestinal toxicity
Inhibiting other key cancer pathways and proteins through crosstalk—Ras, Akt, NF-κB, EGF, VEGF	Increased risk of cancers for which Notch acts as a tumor suppressor
Apoptotic effects	May diminish normal stem cell populations such as in the brain
Cell cycle inhibition	Effect on endothelial cells may stimulate vascular tumor formation over the long term
Senescence?	
Antiangiogenic effects	
Sensitizes to other treatments, such as radiation and chemotherapy	

has not yet been demonstrated. On the other hand, Notch-*activating* agents may have therapeutic potential for these cancers—though with the corresponding risk of increasing risk of other cancers. Despite the potential risks of Notch inhibition, it generally seems well-tolerated and these risks have not appreciably dampened the growing enthusiasm for Notch inhibitors as cancer therapies.

STRATEGIES FOR NOTCH INHIBITION

All current and experimental approaches for inhibiting Notch are discussed below, as well as some theoretical means. These are depicted in Figure 1. Potential benefits and drawbacks of each method are summarized in Table 2.

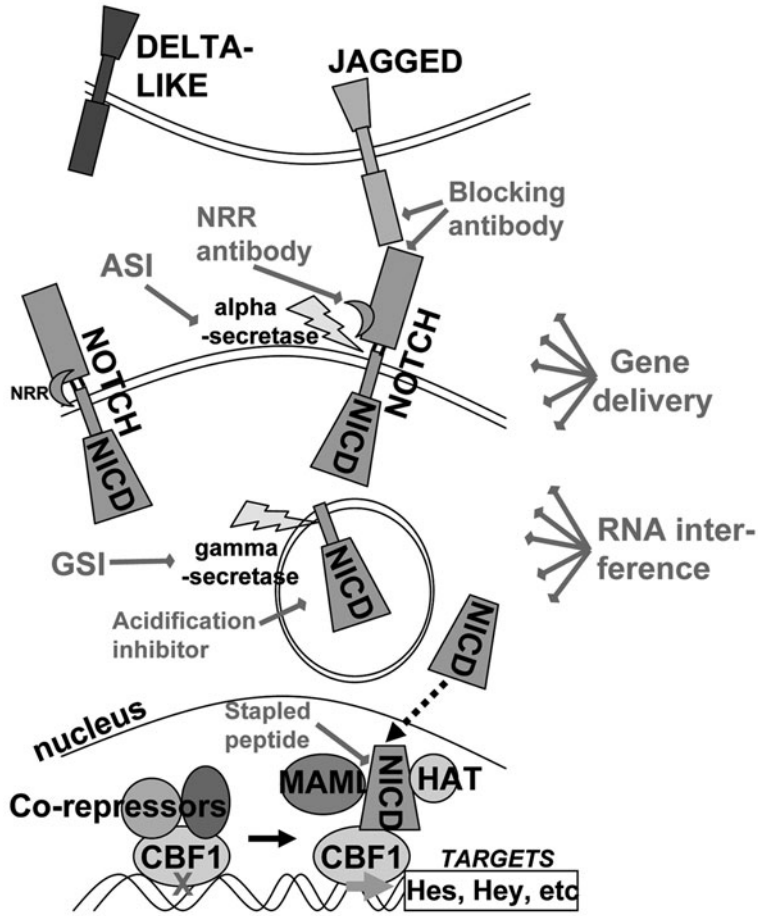


Figure 1. Depiction of Notch pathway and loci at which current and experiemental inhibitors block. Abbreviations: NPR- negative regulatory region; NICD- Notch intracellular domain; ASI- alpha-secretase inhibitor; GSI- gamma-secretase inhibitor; MAML- Mastermind-like; HAT- Histone acetyl-transferase.

Table 2. Benefits and drawbacks of different strategies for Notch inhibition in cancer

	Benefits	Drawbacks
Gamma-secretase inhibitors	Effective Notch inhibitors in most settings. Oral agents. Already in clinical trials. Numerous GSIs already developed or in the pipeline.	Nonspecific. GI toxicity.
Alpha-secretase inhibitors	May be active outside the cell, so not vulnerable to efflux pumps. Oral agents.	Nonspecific. Likely GI toxicity.
Small-molecule blockers	Potential for oral bioavailability and for specificity for individual Notch family members or ligands.	Purely theoretical at this stage.
Endosomal acidification inhibitors	Includes known agents, which may include some already tested in humans. May be oral.	May be highly nonspecific and be similar to GSIs.
Blocking or NRR (negative regulatory region) antibodies	Can be specific for individual Notch family members or Notch ligands. Targeting of individual Notch family members may minimize side effects such as GI toxicity.	Difficult access—may be most useful intravascularly or with local delivery. Large molecule.
Stapled peptide	Highly specific for the Notch pathway. Relatively small molecule.	Difficult access to cells—may be most useful intravascularly or with local delivery.
Delivery of notch-inhibiting genes	May be quite specific.	Difficult access, likely requiring viral or liposomal delivery. Probably inefficient.
Delivery of siRNAs,s hRNAs, or microRNAs	siRNAs/shRNAs can be very potent Notch inhibitors. MicroRNAs are found endogenously and are likely tolerated well by normal cells.	Difficult access, likely requiring viral or liposomal delivery. Likely inefficient but efficiency may be boosted by transduced cells shedding microvesicles taken up by nearby cells.

Small Molecule Inhibitors

Gamma-Secretase Inhibitors

Developing inhibitors of the Notch pathway is complicated by the fact that pathway members themselves do not have enzymatic activity, as it is typically easiest to develop small-molecule inhibitors of enzymes. However, canonical signaling by the Notch pathway does require two enzymatic cleavages that occur following ligand binding to Notch, the first by the alpha-secretase complex and the second by the gamma-secretase complex. These enzymes are amenable to blockade by small-molecule agents and gamma-secretase inhibitors (GSIs) represent the pioneering class of Notch inhibitors

both in the laboratory and in the clinic. It is important to note that gamma-secretase also has other targets besides the four Notch proteins, including the Notch ligands Delta-like and Jagged, APP (amyloid precursor protein), CD44, ErbB4, LRP, syndecan-3, p75 NTR, Apo ER2, DCC, Nectin-1alpha, E-cadherin and possibly N-cadherin.[74-84] GSIs were first developed as potential therapies for Alzheimer's disease and only later were adapted for cancer therapy. This lack of specificity may be problematic for their use in humans, but on the other hand it may be helpful as some of the other GSI targets have themselves been identified as potential targets for cancer therapy.[85-87] A few reports in the literature indicate that at least in some cancer settings the inhibition of Notch is responsible for most of the cytotoxicity of GSIs, evident because restoring expression of the constitutively-active Notch intracellular domain can rescue the cells.[25,88]

Most of the experimental work with Notch inhibitors in the laboratory has been done with GSIs and early clinical trials have already taken place with the Merck GSI MK-0752. The first Phase I clinical trials of MK-0752 were in patients with T-cell leukemia/lymphoma, and other Phase I trials are ongoing in patients with solid tumors and in patients with breast cancer. Stable disease and one response have been observed in patients with high-grade glioma and stable disease was also observed in patients with two other cancer types.[89] A host of other clinical trials are being initiated for patient populations with a variety of cancers, with GSIs either alone or in combination with other agents.

Chemically, a number of structures have been used as the basis for GSIs. The most commonly used is a modified di- or tri-peptide with one to two aromatic hydrocarbon rings included. This has yielded hydrophobic compounds which are cell-permeable and that act as reversible inhibitors of gamma-secretase. In the laboratory, the most widely employed is DAPT (N-[N-(3,5-Difluorophenylacetyl-L-alanyl)]-S-phenylglycine t-Butyl ester) and another frequently-used compound is the structurally similar Lilly GSI L685,458. A structurally different compound which is also available preclinically is compound E ((s,s)-2-(3,5-Difluorophenyl)-acetylamino]-N-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)-propionamide). Another class of GSIs includes diazepine-type structures, with DBZ (dibenzazepine) as an example. Other GSIs are based on an isocoumarin foundation, such as JLK6 (7-amino-4-chloro-3-methoxyisocoumarin) and these can bind and inhibit gamma-secretase irreversibly. One sulfonamide-based GSI, Compound 18 ([11-endo]-N-(5,6,7,8,9,10-hexahydro-6,9-methano benzo[9][8]annulen-11-yl)-thiophene-2-sulfonamide), is reported to be potent.[25,88] Other structures have also been demonstrated to inhibit gamma-secretase activity.[90]

Other drugs in wide usage have been found to have some level of GSI activity, including many nonsteroidal anti-inflammatory drugs (NSAIDs).[91,92] Some of these drugs have anti-cancer and antiangiogenic effects and Notch inhibition via GSI activity might be one mechanism to explain this. NSAIDs have been explored as GSIs particularly in the field of Alzheimer's disease and there have been attempts to derive selective NSAID GSIs which can block amyloid precursor processing but spare Notch processing, to avoid potential side effects from Notch inhibition. One early report described flurbiprofen as such an NSAID.[92] The study of selective GSIs also raises the possibility that agents could be derived that more specifically inhibit Notch processing, sparing other gamma-secretase functions. While GSIs are highly nonspecific and imperfect Notch inhibitors, they still may demonstrate clinical utility and more refined later-generation GSIs may yet emerge.

Other Potential Approaches to Small-Molecule Inhibitors

While attention has focused only on gamma-secretase as a vulnerable point in Notch processing, it may also be feasible to utilize alpha-secretase inhibitors (ASIs) that have been developed for purposes other than Notch inhibition. The alpha-secretase enzymes that cleave Notch are thought to be ADAM-10 and -17 (A Disintegrin And Metalloprotease)[93,94] and inhibitors that block both these ADAMs have been developed.[95] There may be theoretical advantages of an ASI over a GSI; for example, an ASI would not have to enter the cell to act. We are in the process of testing ASIs as Notch inhibitors in cancer.

At the theoretical level, it could be possible to develop small-molecule inhibitors of Notch that act in very different fashions. While inhibition of an enzymatic activity is typically the most straightforward strategy to block a protein or pathway, examples are beginning to emerge of the potential druggability of protein-protein interactions. This was powerfully shown in a recent report in which a small-molecule blocker was derived to interrupt the interaction of the fusion protein EWS-Fli1 with the RNA helicase RHA.[96] This work demonstrated that small molecules could be discovered to disrupt the binding of even highly disordered proteins, lacking alpha helices or beta pleated sheets at the binding domains. Another example of a small-molecule agent blocking a protein-protein interaction is the molecule nutlin, which interferes with p53/MDM2 interaction.[97] A number of protein-protein interactions in the Notch pathway would be logical targets for disruption, including Notch—Notch ligand, Notch intracellular domain (NICD)—CBF1 transcription factor, or NICD—mastermind-like (MAML).

There are likely other points in Notch processing and the Notch pathway that are amenable to blockade. One promising approach was described in a recent poster presentation, but has yet to be published.[98] It relies on the discovery that the gamma-secretase cleavage of Notch occurs not at the cell membrane but in acidic endosomes.[99] Numerous drug compounds with the potential to interfere with endosomal acidification were screened for their ability to reduce Notch activity and this yielded promising hits. The Na+/H+ antiporter Monensin emerged as a potent Notch inhibitor. How acidification inhibitors such as this compare to the GSIs in terms of efficiency and specificity remains to be determined.

Antibody Inhibitors of Notch Activity

Antibody inhibitors remain a prominent means for blocking protein-protein interactions or part of a protein structure and thus represent one modality for disrupting Notch activity. One potential advantage of antibody inhibitors is their specificity, allowing for targeting of individual Notch family members or ligands. Antibodies are large molecules, though and delivery/access to cancer cells could be highly problematic. For certain cancers such as brain tumors, local delivery may be an option, but for most metastatic cancers it is necessary to have efficient systemic distribution. Thus, antibody inhibitors of Notch may be most easily applied toward hematopoietic malignancies or for antiangiogenic uses.

Along these lines, antibodies to the Notch ligand Delta-like-4 represent a highly promising antiangiogenic strategy. As mentioned earlier, Notch signaling via the ligand Delta-like-4 was reported by multiple groups to suppress angiogenic sprouting by endothelial cells. Counter-intuitively, blocking Delta-like-4 with specific antibodies did not promote tumor angiogenesis, but instead led to chaotic, dysfunctional vasculature and subsequent tumor regression.[49,51,55] Importantly, this occurred even in cancer models that

were resistant to VEGF antibodies, an established and powerful antiangiogenic approach already in the clinic. This has prompted an aggressive effort to develop Delta-like-4 antibodies for clinical usage. While this may ultimately prove fruitful, one recent study suggests a potentially significant hazard. Prolonged treatment with a Delta-like-4 antibody in mice led to the development of vascular/endothelial cell-based tumors resembling hemangioblastoma.[100] In hindsight this makes some sense, given the suppressive effect of Delta-like-4/Notch signaling on endothelial cell outgrowth. If this adverse effect is borne out by others as well, it may present a major obstacle to the usage of Delta-like-4 antibodies in the clinic.

Blocking antibodies to Notch or its ligands may serve not only antiangiogenic functions but also directly inhibit cancer cells. A growing number of reports describe the development of antibodies to specific Notch family members, sometimes with different functions. Antibodies to Notch-3 were reported that can either block or stimulate receptor signaling.[101] Some of these antibodies seem to work by binding a region of Notch that obscures the target site for alpha-secretase (the negative regulatory region, or NRR), either by exposing the target site or reinforcing its blockade. This raises the interesting prospect that antibodies could fine-tune Notch activity, increasing or attenuating signaling by individual Notch family members by disparate mechanisms. One exciting report has just emerged in which anti-NRR antibodies were developed that specifically block activity of either Notch-1 or Notch-2.[102] The Notch-1 anti-NRR showed good antitumor effects, but without the gut toxicity associated with combined Notch-1 and Notch-2 inhibition. These Notch isoform-specific antibodies may represent a powerful new modality for cancer therapy, with good effectiveness but decreased toxicity.

Novel Methods of Notch Inhibition

Alternatives for Notch inhibition other than the more standard approaches described above are also becoming possible, in some cases taking advantage of new technologies. One recently-described approach uses a stapled peptide to block interaction of Mastermind-like with the Notch intracellular domain. While protein-protein interactions have generally been considered "undruggable," as discussed earlier, the stapled peptide approach represents a recent development for inhibiting some of these interactions. This strategy was introduced by the Verdine and Korsmeyer labs at Harvard, initially for the development of a Bcl-2 inhibitor.[103] Some protein-protein interactions include an alpha-helix at the interface point, which raised the prospect of using the isolated alpha-helical peptide as a blocker. However, by themselves these peptides are not structurally stable and are also too large and charged to pass the plasma membrane. The Verdine laboratory noted that with the incorporation of two modified residues into the alpha-helical peptide, a hydrocarbon chain could be linked in parallel to the peptide to lock its structure. Some of these peptides retained the ability to block the relevant protein-protein interaction. Surprisingly, the stapled peptides were also found to pass through the plasma membrane, allowing blockade of intracellular protein interactions. A recent report describes a stapled peptide, derived from an alpha-helix in the Mastermind-like (MAML) protein, that blocks canonical Notch signaling.[104] This peptide, termed SAHM1, could have therapeutic potential for Notch-dependent hematopoietic cancers such as T-cell acute lymphoblastic leukemia, or possibly with local delivery to solid tumors.

Genetic strategies for Notch inhibition may also find limited application in cancer therapy, particularly for hematopoietic malignancies or localized tumors, such as in brain

or lung. One form of this could consist of delivery of a gene or pseudogene encoding a Notch-inhibiting protein or peptide. A dominant-negative form of Mastermind-like has been used in the laboratory to inhibit canonical Notch signaling via CBF1[105] and if this could be delivered in vivo it would serve as a highly specific Notch inhibitor. Other genes known to down-regulate Notch could also serve this function, such as the Numb/Numb-like or FBXW-7 genes.[106,107] Agents that up-regulate expression of these endogenous Notch-inhibiting genes could be another means of blocking Notch activity.

Delivery of RNA interference represents a similar strategy for Notch-inhibiting cancer therapy, but possibly one with more potential for clinical success. As with Notch-inhibiting genes, delivery remains the principle hurdle in developing such approaches, but it is relatively less challenging to deliver small oligonucleotides than it is whole genes. Either small interfering RNAs or endogenous or artificial microRNAs could be delivered. Small interfering RNAs (siRNAs) are synthetic 19-27 base pair double-stranded oligonucleotides designed to perfectly match a sequence in a target mRNA and they are incorporated into the cell's RISC complex (RNA-induced silencing complex) with subsequent destruction of the target mRNA. MicroRNAs utilize the same cellular machinery but represent an endogenous form of RNA interference that was discovered more recently than the siRNAs. It is estimated that approximately 1,000 microRNAs exist in the human genome, in both intronic and intergenic regions. The microRNAs originate as small oligonucleotide hairpins that are then processed into mature double-stranded microRNAs similar to siRNAs.[108] However, unlike siRNAs the microRNAs usually target the 3′ untranslated region of target genes and complementarity for the target 3′-UTRs is imperfect. They also more frequently cause translational suppression of targets, but sometimes may cause mRNA cleavage.[109] MicroRNAs each target numerous genes and in general each gene is targeted by more than one microRNA. MicroRNAs thus offer the potential to simultaneously target more than one gene of interest, though the target genes may not be suppressed as efficiently as by siRNAs. For example, the microRNA miR-326 has been shown to target both Notch-1 and Notch-2 and to decrease Notch activity.[110] The tumor-suppressive microRNA miR-34a has also been shown to target Notch-1 and Notch-2[111] and microRNA-206 has been shown to target Notch-3.[112] miR-124 inhibits the important Notch mediator Hes-1.[113] In some cases, transfecting these microRNAs has been shown to not only diminish Notch activity but also to kill cancer cells, and in the case of miR-326 and glioblastoma cells it was suggested that the principle mechanism for cell kill is Notch inhibition.[110] With respect to whether siRNAs or microRNAs would be more successful agents for Notch inhibition and cancer therapy, this remains an open question.

At present the potential delivery modalities for genetic strategies such as RNA interference include viral or liposomal vectors. For these approaches to be successful there will have to be an advance in the technology for delivery, given the requirement that all or nearly all of the cancer cells would have to receive the payload. However, recent studies suggest that this requirement may not be as stringent as once thought, because cancer cells have been shown to shed large amounts of microvesicles that can transmit cytoplasmic contents to nearby cells.[114,115] Results are beginning to emerge that enough siRNAs or microRNAs can be transferred in this fashion to suppress gene expression in neighboring cells. Thus, even if a limited percentage of cancer cells is transfected with a therapeutic vector, the transfected cancer cells may "share" with nearby untransfected cancer cells to produce good results.

CONCLUSION

The Notch pathway has tremendous potential as a new target in cancer therapy. Notch inhibition in cancer cells has the potential to slow cell proliferation, cause apoptosis, induce differentiation and possibly trigger other terminal cell fates such as senescence. These effects are unsurprising given the extensive crosstalk of Notch with major cancer pathways such as Ras, Akt and NF-κB. Importantly, Notch may be a particularly powerful target for the tumor stem cell subset, which is resistant to standard treatments such as chemotherapy and radiation but seems especially sensitive to inhibition of stem cell pathways such as Notch. Even if Notch inhibitors alone do not yield major responses and cures, there is growing evidence that synergy can result from combining Notch inhibition with already-existing treatment modalities such as chemotherapy, radiation and other pathway inhibitors. Optimism for Notch should be tempered somewhat by adverse effects such as gastrointestinal toxicity that are beginning to be observed in clinical trials and no doubt other problems from long-term Notch inhibition remain to be discovered. The field is also hampered by limited existing options for Notch inhibitors; new agents are desperately needed. While gamma-secretase inhibitors are already in clinical trials as Notch-inhibiting agents and are clinically promising, they are highly nonspecific. Other experimental means of Notch inhibition include alpha-secretase inhibitors, peptide or antibody blockers, stapled peptides and genetic strategies such as RNA interference. At present the difficulties in successfully bringing Notch inhibition to the clinic all appear surmountable and there is growing optimism that Notch inhibition will become an exciting new approach to cancer.

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